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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/31, 15/81, C12Q 1/68
C07K 13/00, C12N 1/19
// (C12N 1/19, C12R 1:725, 1:865)

A1

(11) International Publication Number: WO 93/08285

(43) International Publication Date: 29 April 1993 (29.04.93)

(21) International Application Number: PCT/US92/09005

(22) International Filing Date: 26 October 1992 (26.10.92)

(30) Priority data:
782,438 25 October 1991 (25.10.91) US
851,975 16 March 1992 (16.03.92) US

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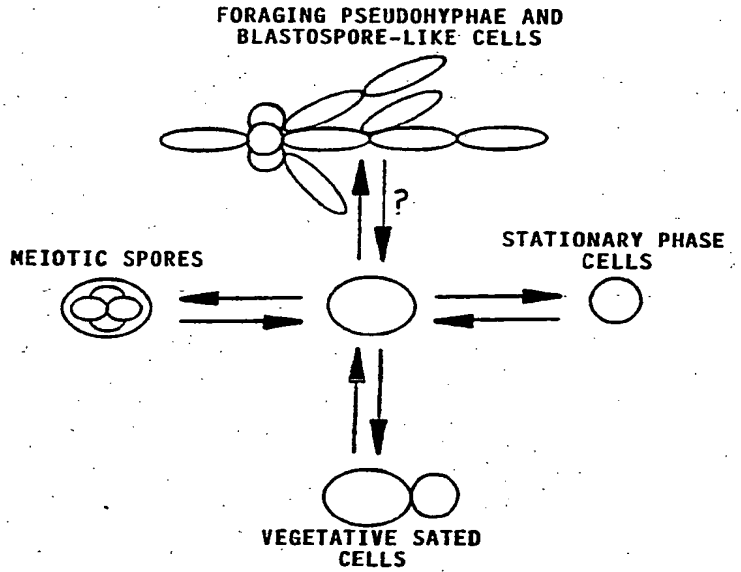
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DIMORPHISM GENES IN FUNGI



(57) Abstract

The present invention relates to a method of identifying a dimorphism gene from a dimorphic fungus; dimorphism genes isolated from dimorphic fungi; the encoded products (proteins, peptides, RNA) which have a role in the dimorphic switch and antibodies raised against proteins or peptides encoded by dimorphism genes. It further relates to agents (drugs) useful for inhibiting the dimorphic switch associated with virulence of fungi and, thus, for causing a dimorphic fungus to remain in its less pathogenic morphological form; a method of inhibiting the dimorphic switch and a method of treating an individual infected by a fungus which undergoes the dimorphic switch. Drugs useful in inhibiting the dimorphic switch can be agents which antagonize activators of dimorphism, agents which stimulate repressors of dimorphism and agents which modulate genes with indirect roles in dimorphism; in each case, the drug causes the dimorphic fungus to remain in its less pathogenic form.

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DIMORPHISM GENES IN FUNGI

Introduction

The polarity of cell division is critical in determining the size and shape of organisms. A cell which undergoes polarized cell division specifically orients its division axis or plane of division with respect to some reference point; a site on the surface of the cell, the position of sibling or ancestral cells, and/or the position of other tissues, organs or structures. For example, oriented cell division is critical in the embryogenesis of both the mouse (Johnson and Maro, Cambridge University Press, pp. 35-65 (1986); Sutherland, et al., Dev. Biol. 137: 13-25 (1990) and the nematode Caenorhabditis elegans (Hyman and White, J. Cell Biol. 105: 2123-2135 (1987); Hyman, A.A., J. Cell Biol. 109: 1185--1193 (1989). The mechanism of directional root growth in higher plants also involves polarized cell division (Gunning, B.E.S., New York, Alan R. Liss, pp. 379-421 (1982).

The yeast S. cerevisiae divides mitotically by budding (Pringle and Hartwell, In The Molecular Biology of

the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, pp. 97-142, Cold Spring Harbor, NY (1981). The bud emerges from a site on the surface of the cell and enlarges while the mother remains relatively constant in size. The mitotic spindle forms along the motherbud axis and, after a set of chromosomes is distributed into the bud, the mother and the bud separate. A chitin plug termed the bud scar is deposited at the site of cell separation and conveniently marks the sites of previous budding events. A single cell can bud many times. The polarity of cell division is defined with respect to the position on the cell surface of previous budding events. Polarized cell division is manifested as two genetically programmed spatial patterns of cell division, axial for a or a cells and polar for a/α cells (Freifelder, J. Bacteriol. 80: 567-568 (1960); Hicks, et al., Genetics 85: 395-405 (1977); Chant and Herskowitz, Cell 65: 1203-1212 (1991)). In the axial pattern the mother and daughter cells bud adjacent to their cell pole that defined the previous mother-daughter junction (see Table 1 for illustration). In the polar pattern a virgin mother's first several buds emerge at the pole opposite the one that defined the junction to its mother (this initial pattern is referred to as unipolar budding); subsequent buds emerge at either this or the opposite pole (Freifelder, J. Bacteriol. 80: 567-568 (1960); Hicks, et al., Genetics 85: 395-405 (1977)) (this latter pattern is referred to as bipolar budding). The biological function of axial haploid budding for mating has been discussed (Nasmyth, K.A., Ann. Rev. Genet. 16: 439-500 (1982), but

to date the function of diploid bipolar budding has remained obscure.

Polar cell division is controlled genetically in S. cerevisiae (reviewed by Drubin, D.G., Cell 65: 1093-1096 (1991)). The current model proposes that budding pattern genes represented by RSR1/BUD1-BUD2/BUD5 (Bender and Pringle, Proc. Natl. Acad. Sci. USA 86: 9976-9980 (1989); Chant and Herskowitz, Cell 65: 1203-1212 (1991); Chant, et al., Cell 65: 1213-1224 (1991); Powers, et al., Mol. Cell. Biol. 9: 390-395 (1991)) are required for selection of the proper bud site and consequently for establishing the proper axis of cell division. RSR/1BUD1, BUD2 and BUD5 convert the default random budding pattern to bipolar and subsequent action of BUD3 and BUD 4 convert bipolar to axial. To explain the observed cell type specificity (diploids are bipolar, haploids axial) an elegant model was proposed (Chant and Herskowitz, Cell 65: 1203-1212 (1991)) that either or both BUD3 and BUD4 are repressed by the repressor alα2 found only in a/α cells. Neither lethality nor alterations in colony morphology were observed in strains that had lost BUD gene function-- random, bipolar and axial budding all lead to the formation of a smooth, hemispherical colony. This is referred to as the unpolarized colonial growth pattern. Dramatic differences in budding pattern seemed to have no effect on growth or colony morphology. It is unclear why yeast have such an elaborate system for determining budding pattern.

Summary of the Invention

The present invention relates to a method of identifying a dimorphism gene from a dimorphic fungus; dimorphism genes isolated from dimorphic fungi; the encoded products (proteins, peptides, RNA) which have a role in the dimorphic switch and antibodies raised against proteins or peptides encoded by dimorphism genes. It further relates to agents (drugs) useful for inhibiting the dimorphic switch associated with virulence of fungi and, thus, for causing a dimorphic fungus to remain in its less pathogenic morphological form; a method of inhibiting the dimorphic switch and a method of treating an individual infected by a fungus which undergoes the dimorphic switch. Drugs useful in inhibiting the dimorphic switch can be agents which antagonize activators of dimorphism, agents which stimulate repressors of dimorphism and agents which modulate genes with indirect roles in dimorphism; in each case, the drug causes the dimorphic fungus to remain in its less pathogenic form.

In particular, the present invention relates to a method of identifying a dimorphism gene from yeast, including Saccharomyces (S.), such as S. cerevisiae and Candida (C.), such as C. albicans; dimorphism genes isolated from dimorphic yeast; the encoded products and antibodies raised against proteins or peptides encoded by a yeast dimorphism gene. Even more particularly, it relates to a dimorphism gene, designated PHD1, isolated from S. cerevisiae; two dimorphism genes, designated CPH1 and PHD5, isolated from C. albicans; dimorphism genes which hybridize to all or a portion of PHD1; dimorphism genes, from other dimorphic fungi, which are the

functional equivalent, in the respective fungi, of PHD1; dimorphism genes which hybridize to all or a portion of CPH1; dimorphism genes, from other dimorphic fungi, which are the functional equivalent, in the respective fungi, of CPH1; dimorphism genes which hybridize to all or a portion of PHD5, and dimorphism genes, from other dimorphic fungi, which are the functional equivalent in the respective fungi of PHD5. Such dimorphism genes can be isolated from any dimorphic fungus, including, but not limited to, plant pathogens (e.g., Ustilago maydis and Ophiostoma ulmi) and human and other animal pathogenic fungi (Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Sporothrix schenckii and Wangiella dermatitidis).

In the method of the present invention of isolating a dimorphism gene, a dimorphism gene is identified by the phenotype it confers on an appropriate host (e.g., S. cerevisiae) when overexpressed in the host. In this strategy, DNA libraries are made from the fungus of interest in vectors designed to overexpress the genes they contain in Saccharomyces cerevisiae. Dimorphism genes from the fungus of interest are identified by the enhanced or suppressed dimorphism phenotype they confer on S. cerevisiae when overexpressed. S. cerevisiae colonies grown on nitrogen starvation medium after one day have a round morphology with few filamentous projections emanating from them. After six days the colonies grow filamentous structures called pseudohyphae and have a striking fuzzy morphology. The present method uses this phenomenon as a way to identify dimorphism genes. Host cells are identified on the basis of the enhanced or suppressed

dimorphism phenotype they exhibit when a dimorphism gene is overexpressed. In the present method, DNA which has a role in the dimorphic switch in a fungus is identified as follows: DNA to be assessed (DNA from a fungus of interest) is digested and inserted into an appropriate plasmid, thereby producing a plasmid library of DNA from the fungus of interest. Using standard transformation methods the library is introduced into a dimorphic diploid yeast strain (such as dimorphic MAT a/α S. cerevisiae) with an auxotrophic marker (e.g., drug resistance marker), thereby producing a mixture of host yeast cells which includes transformed dimorphic yeast and untransformed dimorphic yeast. The resulting mixture is plated on medium containing an appropriate selective agent (e.g., a drug). Transformed dimorphic yeast which contain the auxotrophic marker survive culturing on selective media and those which do not, die. Transformants selected in this manner are screened for the presence of a dimorphism gene in at least one of three ways. In the first screen, the plates are visually screened (e.g., under a microscope) after 3-4 days of growth, and colonies with enhanced pseudohyphal growth are identified by their fuzzy morphology. Normal colonies are symmetrical and round (not fuzzy) under these conditions. Colonies identified as having enhanced pseudohyphal are picked and the plasmids contained in cells in the colonies are isolated, using standard techniques. The plasmids are reintroduced into the original host strain used, in order to verify that they confer the enhanced phenotype. Those that evidence the fuzzy morphology contain a dimorphism gene, which is an activator of dimorphism in a dimorphic fungus (i.e., is

either a positive activator of dimorphism or a gene with a significant, but indirect role in activation of dimorphism).

In a second screen, designed to identify a dimorphism gene which is either a repressor of dimorphism or a gene with a significant but indirect role in dimorphism, plates are scored after a longer period (generally seven days) than for the first screen. After a longer period of time, normal colonies are fuzzy, and those with suppressed pseudohyphal growth are symmetrical and round. As in the first screen, the plasmids in the abnormal colonies are isolated, and reintroduced into the original host strain to verify that they confer the suppressed phenotype. Those that evidence the symmetrical and round morphology contain a dimorphism gene, which is a suppressor of dimorphism in a dimorphic fungus (i.e., is either a suppressor of dimorphism or a gene with a significat, but indirect role in suppression of dimorphism).

In a third screen, designed to identify a dimorphism gene which is either a positive activator of dimorphism or a gene with an important but indirect role in dimorphism, the plates are rinsed, and then screened under a dissecting microscope for colonies imbedded in the agar. Normal colonies do not remain in the agar. Imbedded colonies are further screened for colonies with filaments of cells; filamentous grouth indicates the activation of a dimorphism gene. As in the first two screens, the plasmids in these colonies are isolated and reintroduced into the original host strain to verify that they confer the imbedded, filamentous phenotype.

Using these methods of screening, three genes with significant roles is dimorphism have been isolated: the gene PHD1 from S. cerevisiae, and the genes CPH1 and PHD5 from C. albicans. These genes have been cloned and sequenced. Other dimorphism genes from any dimorphic fungus may be isolated with these methods, and then cloned and sequenced by standard methods. Primers or fragments generated from the sequence of an identified dimorphism gene, such as PHD1, CPH1, or PHD5, can be used to isolate other dimorphism genes homologous to the previously identified gene, with known hybridization methods and amplification methods (e.g., PCR). The proteins encoded by identified dimorphism genes can also be isolated, and antibodies raised against such proteins. Antibodies which recognize an identified dimorphism gene product can then be used to identify other products encoded by additional dimorphism genes.

The current invention makes it possible, through the identification of dimorphism genes, to alter (inhibit or enhance) the dimorphic transition or cell change, in yeast as well as in other organisms in which homologous genes have similar roles. Alteration can be effected by introducing into cells agents or drugs (e.g., peptides, small organic or inorganic compounds, oligonucleotides, plasmid-based constructs which express anti-sense RNA to said genes) which alter the dimorphic change or passage from one form to the other (nonpathogenic to pathogenic) by direct or indirect effect on the gene or gene product. Such agents can be used as antifungal agents.

Brief Description of the Drawings

Figure 1 shows results of genetic and physiological characterization of S. cerevisiae pseudohyphal growth.

Figure 2 shows results of SEM analysis of starvation-induced cell morphology changes.

Figure 3 shows the invasiveness of S. cerevisiae pseudohyphae.

Figure 4 is a restriction endonuclease map of the SHR3 region and SHR3 plasmid inserts. The restriction map of the 13 kb BamHI clone (pPL154) and the subsequent subcloned fragments containing the SHR3 coding region is indicated as a solid arrow (pPL210). The 1.1 kb HindIII URA3 fragment inserted into the HindIII site of the engineered SHR3 deletion plasmid pPl216 is shown as a cross hatched box. Restriction endonuclease sites are labelled as follows: A, AccI; B, BamHI; E, SpeI; H, HindIII; R, EcoRI; P, PstI; S, SalI. The AccI sites enclosed in parentheses were inactivated during the subcloning process.

Figure 5 is the nucleotide sequence of the SHR3 gene and deduced amino acid sequence (SEQ ID NO. 1)

Figure 6 is a hydropathy plot of the deduced SHR3 amino acid sequence. Hydropathy values were obtained using the Kyte and Doolittle algorithm using a window size of 12 amino acid residues.

Figure 7 is a graphic representation of histidine and arginine uptake in intact and Cu^{2+} treated yeast cells. Exponentially grown cells in YPD media were harvested and washed twice. Cells suspended in 10 mM MES-Tris pH 6.4, mM $MgCl_2$, 0.6 M sorbitol supplemented with 10 μCi of [^{14}C]-histidine (0 083 mCi mmol^{-1}) (a) or [^{14}C]-arginine

(0.25 mCi $mmol^{-1}$) (b) per ml. Subsamples were withdrawn and filtered at the times indicated. For histidine uptake, at 5 min the culture was split and 800 μM $CuCl_2$ was added. For arginine uptake, at 10 min the culture was split and 800 μM $CuCl_2$ was added. Symbols: •, untreated; □, $CuCl_2$ treated. Panel 1, wildtype cells; Pane 2, shr3-3 cells.

Figure 8 is a schematic representation of developmental pathways of diploid yeast cells.

Figure 9 shows results of genetic and physiological characterization of S. cerevisiae and pseudohyphal growth.

Figure 10 shows results of $RAS2^{val19}$ induction of pseudohyphal growth.

Figure 11 shows the results of production of blastophore-like cells by S. cerevisiae pseudohyphal cells.

Figure 12 is the nucleotide sequence of the PHD1 gene and deduced amino acid sequence (SEQ ID NO.:2).

Figure 13 shows β-galactosidase activity in SR3 (PLAS1-7D) and shr3-23 (PLAS23-4B) strains transformed with GCN4-LacZ plasmids with: GCN4-LacZ under general control (p180) (a); and gch4-LacZ constitutively expressed (p227) (b). β-galactosidase activities were determined in transformants grown in complete synthetic media minus uracil for repressing conditions (Repressing), and in strains grown under depressing conditions in complete synthetic media minus uracil and histidine (DR-his).

Figure 14 is the nucleotide sequence of the CPH1 gene (SEQ ID NO.:3).

Figure 15 is the nucleotide sequence of the PHD5 gene (SEQ ID NO.:4).

Detailed Description of the Invention

The present invention relates to a method of inhibiting (totally or partially) the dimorphic switch undergone by fungi, particularly yeast. As a result of work described herein, the ability of a fungus to undergo the switch from yeast-like to filamentous growth and from filamentous to yeast-like growth (i.e., the dimorphic switch which is characteristic of fungi which exhibit fungal dimorphism) can be inhibited. Because of the role of dimorphism in virulence of pathogenic fungi, the work described herein serves as the basis for reducing the adverse effects of a pathogenic fungus which undergoes the dimorphic switch and for causing the dimorphic fungus to remain in its less pathogenic morphological form. In particular, the dimorphic switch undergone by yeast is described herein and can be inhibited. The present work, thus, provides the basis for prevention and/or treatment of the adverse effects of pathogenic fungi, including, but not limited to yeast, which are pathogenic to plants and animals, including humans.

Applicants have discovered, as described herein, two different types of genes, each of which encodes a different yeast protein and has a significant role in the dimorphic switch in yeast. First, they have identified yeast genes which, when overexpressed in yeast cells, causes enhanced pseudohyphal growth. One of these genes, isolated from S. cerevisiae and designated PHD1 (pseudohyphal determinants), has been further characterized. Results of the genetic and sequence analysis suggest that PHD1 encodes a regulatory protein which controls pseudohyphal growth and has significant homology to proteins which

are transcriptional regulatory proteins and regulate development of diverse fungi. The fact that PHD1 localizes to the nucleus supports the idea that it is a transcriptional regulatory protein and the sequence homologies suggest that it is a DNA binding transcription factor. Two dimorphism genes, designated CPH1 and PHD5, have been isolated from the human pathogen C. albicans by the same approach as was used to isolate PHD1. Second, Applicants have also identified a yeast gene, designated SHR3, which encodes a protein, located in the endoplasmic reticulum, which is required for the function of multiple different yeast amino acid permeases and, thus, for amino acid transport. They have shown that in yeast cells without SHR3 (SHR3 mutants), amino acid permeases accumulate in the endoplasmic reticulum and are not transported to the plasma membrane. Further, they have shown that the endoplasmic reticulum export block is specific for this class of permeases; that SHR3 mutations impair proline transport and induce starvation responses in yeast cells in which they are present; and that the amino acid transport defects of such mutants are reflected in increased filamentous or pseudohyphal growth. These observations suggest filamentous growth is a manifestation of a diploid specific developmental pathway which is induced by conditions of nutrient limitation; that yeast cells require a minimum concentration of permeases in plasma membranes to assess accurately the extra cellular nutrient levels; and that nitrogen source availability regulates the dimorphic transition. That is, if the plasma membrane permease concentration is too low or nitrogen source

availability is limited, the yeast cells enter the pseudohyphal phase inappropriately.

Thus, as a result of the work described herein, fungal dimorphism genes, particularly yeast dimorphism genes, their encoded products (RNA, proteins, polypeptides), and agents which bind the genes of an encoded product (e.g., oligonucleotides, antibodies, peptides, proteins, peptide-like agents, small organic molecules) are available. Also available are methods of identifying additional fungal dimorphism genes; methods of interfering with the expression of such genes; methods of interfering with the encoded products; methods of inhibiting the dimorphic switch (and, thus, of reducing the adverse effects of pathogenic fungi in which the switch is associated with development of virulence); and agents (referred to as drugs) useful in such methods.

The present work also makes available fungal genes, particularly yeast genes, which encode proteins required for permease function and, thus, amino acid transport; their encoded products (RNA, proteins, polypeptides); and agents which bind the genes or an encoded product (e.g., olignucleotides, antibodies, peptides, proteins, peptide-like agents, small organic molecules). Further, it makes available methods of identifying additional fungal genes (referred to as SHR3-equivalent genes) which encode similar (SHR3-equivalent) proteins; methods of altering, particularly enhancing, expression of SHR3 or SHR3-equivalent genes; methods of interfering with the dimorphic switch (and, thus, of reducing the adverse effects of pathogenic fungi in which the switch is associated with

development of virulence); and agents (referred to as drugs) useful in such methods.

The following is a description of the identification, isolation and characterization of genes which have a role in the dimorphic switch which occurs in fungi, particularly genes which have a role in the dimorphic switch in yeast and of applications possible as a result of the present work, which provides not only an understanding of the dimorphic switch which occurs in fungi, but also novel means for effectively inhibiting the switch. Applicants' assessment of the effect of environmental factors on pseudohyphal growth of S. cerevisiae and the physiological role that pseudophyhal growth plays in the organism's functions or activities is also described below.

Yeast Cell Growth

The ability of some fungi to switch from yeast-like to filamentous growth and from filamentous to yeast-like growth is referred to as fungal dimorphism. Many fungi pathogenic to plants and animals are dimorphic including the plant pathogens Ustilago maydis and Ophiostoma ulmi and the important human pathogens Candida albicans, Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Sporothrix schenckii, and Wangiella dermatitidis. The virulence of some dimorphic fungi is known to be related to their ability to undergo the dimorphic switch.

A yeast cell grown on standard media multiplies until it forms a visible structure, an approximately hemispherical colony with a smooth circular outline. This morphology is strikingly homogeneous, with little vari-

ation from colony to colony. The second distinct mode of proliferation, pseudohyphal growth, results from a reiterated pattern of unipolar cell division. A pseudohypha is defined as a "fragile chain of cells (usually yeasts, which have arisen by budding and have elongated without detaching from adjacent cells), with morphological characteristics intermediate between a chain of yeast cells and a hypha" (Evans and Richardson, Oxford, Information Press Ltd., 1989). Pseudohyphal growth in S. cerevisiae is a unique type of polarized cell division that requires unipolar budding and a change in cellular morphology that results in the formation of macro-scopic structures emanating away from the colony into unpopulated substrate. Reiteration of unipolar cell division by pseudohyphal cells leads to the formation of an asymmetric polarized colony. In polarized colonies, which resemble colonies formed by filamentous fungi, the pseudohyphae radiate outward in all directions (Figure 1).

As described below, Applicants have shown that under conditions of nutrient limitation, diploid yeast strains enter a new developmental pathway which involves changes in cell shape, the pattern of cell division and gene expression. In particular, as described herein, it has now been shown that S. cerevisiae make pseudohyphae under conditions of nitrogen starvation and that the pseudo-hyphae can invade the medium on which the yeast is grown. This pseudohyphal pathway permits cells to forage for nutrients at a distance from their initial location. The invasive nature of the type of growth exhibited under conditions of nutritional deprivation is similar to that of pathogenic dimorphic fungi and has implications for

diseases in both plants and animals. Constitutive activation of RAS2 or deletion of SHR3 enhances the pseudohyphal phenotype. Random budding induced by RSR1/BuDI abolishes pseudohyphal growth. As further described, the consequences of mutations in the SHR3 gene on regulation of amino acid metabolism, general amino acid control and cellular response to starvation have been assessed, the SHR3 gene has been cloned and S. cerevisiae SHR3 mutants in which histidine resistance is affected have been produced.

Isolation of dimorphism genes in S. cerevisiae and in C. albicans

Another outcome of the present work is identification of genes, including PHD1 of S. cerevisiae and CPH1 and PHD5 of C. albicans which affect or participate in the dimorphic change or transition from one form to the other. As referred to herein, the term dimorphism gene includes genes from other fungi (including but not limited to genes from other yeast) which 1) are a) identified by the assay described herein in which the phenotype conferred on S. cerevisiae by overexpression of candidate genes is assessed (see Isolation of Dimorphism Genes and Example 5); or b) sufficiently homologous to all or a portion of a dimorphism gene described herein (PHD1, CPH1, PHD5) to hybridize under the conditions (low stringency or standard hybridization conditions, as desired) and 2) confer a phenotype characteristic of a dimorphism gene when assessed using the assay method described herein (Isolation of Dimorphism Genes and Example 5). Their encoded products are referred to herein as dimorphism gene products or proteins encoded by a

dimorphism gene. Such proteins can be isolated proteins encoded by a dimorphism gene or recombinantly produced proteins encoded by a dimorphism gene.

That is, genes which are likely to be targets useful in controlling or preventing the pathogenicity of yeast and other organisms in which they function have been identified. Such genes and/or their gene products can be inhibited, directly or indirectly, by use of a variety of agents, such as peptides, anti-sense nucleic acid sequences and small organic or inorganic molecules. In addition, these genes can be used in an assay to screen antifungal compounds for their effect on pseudohyphal growth, as well as to identify genes in other organisms which are required for pathogenesis.

A dimorphism gene from S. cerevisiae called PHD1 has been isolated. A genomic library of S. cerevisiae was generated, and used to transfect a dimorphic strain of S. cerevisiae. The cells were plated on selective media. After several days, surviving colonies (i.e., those which have incorporated plasmids) were plated on low-ammonium media. Three to four days later, colonies were examined under a dissecting microscope, to select those displaying the fuzzy morphology characteristic of pseudohyphal growth, rather than the symmetrical and round morphology of yeast-like growth. The plasmids from the selected fuzzy colonies were isolated using standard techniques, and then retransfected into the host strain of S. cerevisiae to ensure that the plasmid did confer the pseudohyphal phenotype. The gene PHD1 was isolated from plasmids which generated pseudohyphal growth. The nucleotide sequence of PHD1 has been determined, as shown

in Figure 12. Conceptual translation of the predicted 1.1 kb PHD1 open reading frame predicts a polypeptide of 40.6 kilodaltons; Western blotting experiments using antiserum which recognizes an epitope tagged version of the PHD1 protein shows that it is about 41 kilodaltons in size. PHD1 is 70% identical over 100 amino acids to the stunted gene from the filamentous fungus Aspergillus nidulans. stunted is a regulator of conidiophore morphogenesis and the sexual cycle. PHD1 also has significant homology to SWI4 from S. cerevisiae and cdc10+ from Schizosaccharomyces pombe whose protein products are both known to be constituents of transcription factors. Consistent with these homologies to transcriptional regulatory proteins, epitope tagged PHD1 protein localizes to the nucleus in indirect immunofluorescence experiments.

The PHD1 gene sequence can be used to isolate genes homologous to PHD1 from other dimorphic fungi. These genes are good candidates for antifungal drug targets, since they will probably regulate dimorphism. This can be done by screening DNA libraries made from the fungus of interest with hybridization probes derived from the PHD1 coding sequence. Alternatively, the amino acid sequence of PHD1 can be used to design degenerate oligonucleotides for PCR and PHD1 homologs can be identified by using these oligonucleotides in PCR reaction with genomic DNA from any fungus. If deletion of PHD1 produces a characteristic phenotype, yeast strains deleted for PHD1 can be used to identify functional homologs from other fungi by complementation of this phenotype by heterologous genes from DNA or cDNA libraries made in yeast vectors from the

fungus of interest. The PHD1 gene itself can be used as an antifungal drug target.

Furthermore, the protein encoded by the PHD1 gene can be isolated, and antibodies raised to the protein can be used to affect pseudohyphal growth in S. cerevisiae. Similarly, antibodies can be raised to proteins or in other fungi in which a gene or protein, homologous to PHD1 or its protein, has been identified. A strain that is constitutively in the pseudophyphal growth made has also been isolated. This strain is useful for the isolation of proteins or RNAs that are expressed in pseudohyphal cells which may be involved in dimorphism, as well as for a screen for drugs.

Methods identical to those used to identify PHD1 in the nonpathogenic fungus, S. cerevisiae, were used to identify pseudohyphal genes in the pathogenic fungas C. albicans. Genes from C. albicans can be expressed in S. cerevisiae, as some C. albicans promoters function in a S. cerevisiae system. A genomic library of a mutant strain of C. albicans was transfected into a dimorphic strain of S. cerevisiae, and those colonies displaying pseudohyphal growth were selected. The plasmids from those colonies were isolated and retransformed into the host strain to ensure that the plasmids conferred the pseudohyphal phenotype. The gene CPH1 was isolated from the plasmids which caused enhanced pseudohyphal growth. CPH1 has been sequenced, as shown in Figure 14. In the same manner, a genomic library from wild-type C. albicans was used to isolate the gene PHD5, the sequence of which is shown in Figure 15. Details of the methods used to isolate these genes are further described in Examples 6 and 7.

Isolation Of Dimorphism Genes

The strategy to isolate dimorphism genes targets three classes of dimorphism genes: genes which activate dimorphism, genes which repress dimorphism, and genes with significant but indirect roles in dimorphism. All three classes of genes are potential targets for antifungal drugs. In this strategy DNA libraries are made from the fungus of interest in vectors designed to overexpress the genes they contain in Saccharomyces cerevisiae. Dimorphism genes from the fungus of interest are identified by the enhanced or suppressed dimorphism phenotype they confer on S. cerevisiae when overexpressed. S. cerevisiae colonies grown on nitrogen starvation medium after one day have a round morphology with few filamentous projections emanating from them. After six days the colonies grow filamentous structures called pseudohyphae and have a striking fuzzy morphology. This phenomenon can be used to identify dimorphism genes. Some positive activators of dimorphism when overexpressed cause one to four day old colonies to have a fuzzy appearance because they have activated dimorphic switches in the cells in the colony. Some negative activators of dimorphism prevent six day old colonies from having a fuzzy appearance because they prevent the cells in the colony from undergoing dimorphic switches. Genes with indirect roles in dimorphism, depending upon their function in the cell, when overexpressed may enhance or suppress dimorphism. The details of this method are further explained in Example 5.

Using these methods, dimorphism genes from any fungus which is pathogenic to plants or to animals, especially humans, can be isolated. Such fungi include, but are not

limited to, plant pathogens (e.g., Ustilago maydis and Ophiostoma ulmi) and human and other animal pathogenic fungi (Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Sporothrix schenckii and Wangiella dermatitidis).

To isolate a dimorphism gene in the fungus under investigation, a genomic or cDNA library is created, as described above; a dimorphic fungus is transfected with the library; and the resulting colonies are screened for colonies which exhibit an enhanced or suppressed pseudohyphal phenotype.

Genes isolated by these methods can be sequenced by appropriate methods, as were PHD1, CPH1, and PHD5. The encoded products of an isolated pseudohyphal gene can be determined, and targeted by agents to combat pathogenic growth. Antibodies raised to the encoded protein can be generated by standard methods, and may be either polyclonal or monoclonal; these antibodies can be used to affect the dimorphic switch in pathogenic fungi by interfering with the pseudohyphal pathway. Inhibitors other than antibodies can also be generated; such inhibitors include oligonucleotides, peptides and DNA fragments, which are designed to interfere with the pseudohyphal gene, with its mRNA, or with its encoded product. Such inhibitors and antibodies can be used to inhibit fungal growth in an individual afflicted with a fungal infection. The agent (antibody, peptide, DNA fragment, or other compound designed to interfere with a pseudohyphal gene, its mRNA, or its product) is administered to the individual in a therapeutically effective amount, defined as an amount sufficient to limit

or eliminate pseudohyphal growth of the fungus. The agent can be administered intravenously, topically, orally, rectally, nasally, buccally, vaginally or by inhalation spray. The method by which the agent is administered will depend in part on the location of the fungal infection. The form in which the agent is administered (e.g., tablet, solution, emulsion, lotion) will depend in part on the route by which the agent is administered.

Amino Acid Uptake and Pseudohyphal Growth

In addition, the current invention pertains to a method of influencing pseudohyphal growth in a fungus, by altering the amino acid uptake of the fungus. In particular, the invention includes a gene isolated from S. cerevisiae which confers resistance to otherwise toxic levels of histidine. Spontaneous mutants resistant to inhibition by high levels of histidine were isolated and characterized; from these mutants the gene SHR3 was isolated, cloned, sequenced and mapped. Strains of S. cerevisiae with mutant SHR3 genes demonstrate altered pseudohyphal growth patterns. The SHR3 gene, PCR primers generated from its sequence, and the protein encoded by SHR3 can be used to isolate other genes influential in dimorphic transition. Investigation of SHR3 activity thus has provided a greater understanding of the role of amino acid uptake in pseudohyphal growth.

The Dimorphic Switch to Pseudohyphal Growth Is Induced by Nitrogen Starvation

The transition from unpolarized colonial growth to pseudohyphal growth occurs on agar based synthetic growth medium deficient in nitrogen. Wild type cells form pseudohyphae on standard minimal medium containing low levels of ammonia (SLAHD) or proline as sole nitrogen source (SPHD). On the low ammonia medium all of the wild type colonies form pseudohyphae (CGX31), whereas on proline medium (SPHD) small regions of pseudohyphal growth are apparent in about a quarter of the colonies (CGX31). CGX31 does not form pseudohyphae when grown on standard ammonia based medium (SD) or media with the same composition as SPHD but containing as sole nitrogen source(s) standard levels of ammonia (SAHD), arginine (SRHD), proline and ammonium sulfate (SPAHD), or proline and arginine (SPRHD).

Of all strains tested, those with the Σ1278b background undergo the most uniform and easily controlled transition from unpolarized to pseudohyphal growth on both low ammonia and proline medium. Many laboratories commonly use strains derived from this background (Grenson et al., Biochim. Biophys. Acta. 127: 325-338 (1966); Brandriss and Magasanik, J. Bacteriol. 140: 498-503 (1979)) because they are extremely sensitive to the ammonia repression of nitrogen assimilation pathways (Rytka, J., J. Bacteriol 121: 562-570 (1975); Wiame et al., Adv. Microb. Physiol. 26: 1-88 (1985)). Σ1278b and its derivatives cross well with other standard laboratory strains such as S288C (Siddiqui and Brandriss, Mol. Cell. Biol. 8:4634--4641, 1988) and comprise part of the set of interbreeding

laboratory isolates known collectively as Saccharomyces cerevisiae.

Mutations in the SHR3 Gene Enhance Pseudohyphal Growth

Diploid strains homozygous for mutant loss of function SHR3 alleles in several backgrounds produce a more uniform, prolific and extended transition to the pseudohyphal growth pattern on SPHD medium than wild type strains. Mutations in the SHR3 gene lead to reduced uptake of many amino acids including proline, as described herein. This reduction in proline uptake probably preceived starvation for nitrogen and accounts for the reduced growth on SPHD of shr3 strains as compared with SHR3 strains. Strains containing shr3 mutations also show more extensive and exaggerated pseudohyphal growth than wild type on low ammonia medium. The enhanced pseudohyphal growth of a $Shr3^-$ strain (CGX19) on low ammonia can be explained if ammonia uptake, like amino acid uptake, is impaired. On medium containing standard levels of ammonium sulfate as sole nitrogen source, $Shr3^+$ (CGX31) and $Shr3^-$ (CGX19) cells grow at similar rates, and neither strain forms pseudohyphae. The fact that CGX19 fails to form pseudohyphae when proline medium (SPHD) contains ammonia (SPAHD) supports the idea that it is nitrogen starvation that induced pseudohyphal formation. To prove it is loss of function of SHR3 that is responsible for pseudohyphal growth, CGX19 (MATa/α shr3-102/shr3-102 ura3-52/ura3-52) was transformed with a centromere based plasmid containing either no insert (pRS306) or the SHR3 (pPL210) gene. Transformants containing pRS306 (CG64) showed pseudohyphal growth identical to that exhibited by

CGX19, whereas the pPL210 transformants (CG62) did not. Only a minority of colonies of diploid cells homozygous for shr3 in a S288C background have pseudohyphae and the number of pseudohyphae per colony is much lower than that observed in a comparable Shr3$^-$ Σ1278b strain. Diploid cells derived from a shr3 S288C parent and a shr3 Σ1278b parent show the pseudohyphal growth characteristic of Σ1278b Shr3$^-$ diploids.

Activation of the RAS2 Protein Enhances Pseudohyphal Growth

Strains carrying the dominant RAS2^{val19} mutation show greatly enhanced pseudohyphal growth. The RAS2^{val19} mutation results in a constitutively activated RAS signal transduction pathway and consequent elevated intracellular cAMP levels (Toda et al., Mol. Cell. Biol. 7: 1371-1377 (1985)). Strains with an activated RAS/cAMP pathway are very sensitive to nitrogen starvation (Toda et al., Cell 40: 27-36 (1987)). Since the RAS2^{val19} mutation is dominant (Kataoka et al., Cell 37: 437-445 (1984); Powers et al., Mol. Cell. Biol. 9: 390-395 (1989)), its effects on growth could be tested by introducing it into our standard RAS2$^+$ Σ1278b strains by transformation. CGX31 transformed with a plasmid containing RAS2^{val19} (YCpR2V), kindly provided by Wigler, exhibits greatly enhanced pseudohyphal growth. The same SHR3$^+$ strain (CGX31) transformed with vector (YCp50) alone shows only low frequency pseudohyphal growth on SPHD medium. The pseudohyphal growth of CGX31 carrying the RAS2^{val19} mutation occurs in a SHR3$^+$ background where growth on SPHD is much better than that of SHR3$^-$ strains.

Thus, it is not simply the poor growth of SHR3⁻ strains on SPHD that results in pseudohyphal development.

An activated RAS pathway in S. cerevisiae either directly or indirectly results in enhanced pseudohyphal growth. MATa/α wild-type diploids carrying the $RAS2^{val19}$ mutation (analogous to the missense mutation found in some transforming alleles of mammalian RAS (Barbacid, M., Ann. Rev. Biochem. 56: 779-827 (1987); Powers et al., Mol. Cell. Biol. 9: 390-395 (1989))) undergo enhanced pseudohyphal growth on proline medium and exhibit pseudohyphal growth on rich YPD medium. RAS2 mutants have perturbed responses to environmental stresses (Kataoka et al., Cell 37: 437-445 (1984); Tatchell et al., Nature 309: 523-527 (1984); Tatchell et al., Proc. Natl. Acad. Sci USA, 82: 3785-3789 (1985)). The RAS pathway is thought to regulate certain stress responses in yeast (reviewed by Broach and Deschennes, Adv. Cancer Res. 54: 79-139 (1990)). Results described herein suggest that one role of the RAS pathway may be to regulate dimorphic transition of S. cerevisiae to pseudohyphal growth. A preferred model is one in which nitrogen starvation induces the RAS pathway and signals the cell to enter the pseudohyphal pathway. Alternatively, a constitutively activated RAS pathway may perturb proline assimilation pathways in a way that enhances pseudohyphal growth. The present work suggests, but does not prove, that cAMP regulates S. cerevisiae dimorphism because RAS is thought to modulate other signalling pathways in yeast (Kaibuchi et al., Proc. Natl. Acad. Sci. USA 83: 8172-8176 (1986)). Evidence exists that cAMP plays a role in the regulation of the dimorphism

of several fungi (Shepherd, M.G., Curr. Top. Med. Mycol. 2: 278-304 (1988).

Pseudohyphal Growth Is a Diploid Specific Pathway

Diploid, but not haploid, S. cerevisiae strains give rise to pseudohyphae. The effect of ploidy and the genotype at the mating type locus on pseudohyphal growth was studied using a congenic set of yeast strains carrying a mutant allele of SHR3. The morphology of the diploid CGX19 was compared with its two haploid parents carrying the shr3-102 mutation. No SHR3-102 haploids analyzed manifested pseudohyphal growth; all formed typical hemispherical unpolarized colonies on SPHD. MATa/a shr3102/-shr3-102 (CG85) and MATα/α shr3-102/shr3-102 (CG67) isogenic derivatives of CGX19 MATa/α SHR3102/SHR3-102) also do not form pseudohyphae on SPHD; instead they form hemispherical colonies. In addition, a and α haploid strains carrying the $RAS2^{val19}$ allele (CG73 and CG75, respectively) do not form pseudohyphae whereas the a/α diploid resulting from crossing these haploids does. The cell type specificity of pseudohypal growth is controlled in part by the alleles of the mating type locus.

Pseudohyphal Growth Results from Unipolar Cell Division

The unipolar cell divisions that characterize polar diploid budding are critical for the elaboration of pseudohyphal growth. Virgin cells are defined as those that have had no daughters and sated cells as those growing vegetatively on rich medium. The budding pattern of virgin sated CGX19 cells or of virgin CGX19 cells growing in pseudohyphae was observed by time lapse photo-

microscopy. Results of a time lapse experiment where the development of a pseudohypha was monitored for 6 hours showed that serial reiteration of unipolar budding by terminal pseudohyphal cells results in polarized chain elongation. It was also seen that the second bud of a virgin terminal cell initiates a new lateral chain oriented at an angle from the main lineage.

Budding pattern was assayed quantitatively by determining the site of emergence of the first and second buds of virgin pseudohyphal and sated cells by time lapse observation (Table 1).

TABLE 1

	First Buds		Second Buds	
	Pseudohyphal	Sated	Pseudohyphal	Sated
Cell Divisions	90	69	90	69
Free End	100%	100%	90%	73%
Birth End	0%	0%	10%	27%

Budding Pattern of Pseudohyphal and Sated a/α Shr3⁻ Cells: Time lapse photography was used to determine bud site selection in both pseudohyphal and sated sells as described in the experimental procedures.

Following the conventions of Freifelder, D., (J. Bacteriol. 80: 567-568 (1960)), the pole of a bud which contacts its mother cell is called the birth end and the opposite pole the free end. The first bud of 90 virgin terminal pseudohyphal cells and 69 virgin sated cells of strain CGX19 emerged without exception on the free end of its mother cell. The first bud of a diploid is, therefore, a good marker for the free end of this cell. The second bud of 90 virgin terminal pseudohyphal cells

emerged in 90% of the cases again on the free end of its mother cell after two doubling times had elapsed. The shape of these cells together with their immobility in the agar matrix permitted easy identification of a cell's poles. The proliferation of ancestral cells prevented scoring of events at the birth end in the other 10% of the cases where no bud was present at the free end after two doubling times. The second bud of each of 69 sated cells emerged from the mother cell's free end, which is identified in this case as the same cell pole from which the first bud emerged, 73% of the time and from the birth end, defined as the opposite pole, 27% of the time. Clearly the first bud of virgin CGX19 cells emerges in a unipolar manner from the free end regardless of the cell's growth mode. The second bud also emerges unipolarly in the majority of cell divisions.

It is important to note that in some lineages lateral budding was completely absent whereas apical growth continued (see Figure 3B). In other words, daughters divided for several divisions while the mother cells did not, suggesting that in these lineages cell division may be repressed after a cell gives birth to its first daughter. In the fungal literature this phenomenon is known as apical dominance (Rayner, A.D.M., Mycologia 83: 48-71 (1991)).

The Cells of the Pseudohypha Are a Morphologically Distinct Cell Type

The dimensions of pseudohyphal and sated cells of the same genotype were compared. In the first experiment SHR3⁻ (CGX19) cells were grown on YPD or SPHD + uracil

media. Cells taken from the surface of the agar were prepared for scanning electron microscopy (SEM). Figure 2 shows scanning electron micrographs of a typical ellipsoidal CGX19 cell from the UPD plate as well as a CGX19 pseudohyphal cell from the SPHD + uracil plate. To be certain that the surface grown cells in SEM micrographs were representative of cells in invasive pseudohyphae, the dimensions of the latter were also measured by light photomicroscopy (Table 2).

TABLE 2

Dimensions of Pseudohyphal Cells, Sated Cells and Blastophore-Like Cells

Strain	Cell Type	Medium
CGX19	Pseudohyphal (SEM)	SPHD+U
CGX19	Sated (SEM)	YPD
CGX19	Pseudohyphal (LM)	SPHD+U
CGX19	Sated (LM)	YPD
CGX19	Blastospore-Like (LM)	SPHD+U

Strain	Length (μm)	Width (μm)	Length/Width
CGX19	6.7±1.0(3)	1.9±0.1(3)	3.5
CGX19	4.2±0.4(7)	3.0±0.2(7)	1.4
CGX19	9.2±1.7(11)	2.7±0.3(11)	3.4
CGX19	5.7±0.8(19)	3.9±0.4(19)	1.5
CGX19	5.6±0.5(10)	4.4±0.4(10)	1.3

Table Legend: CGX19 cells (MATa/α ura3-52/ura3-52 shr3--102/shr3-102) were measured in all cases. Cell dimensions are based on scanning electron and light photomicrographs as described in the experimental section. Cell length is the length of the longest axis of the cell. Cell width is the width of the cell at the midpoint of its longest axis. The axial ratio is the average cell length divided by the average cell width. The tabulated values are averages with standard deviations listed. The number of cells measured for each table entry appears in parentheses after the standard deviation.

Given the difference in imaging methods, the two sets of measurements agree well and give similar axial ratios. Pseudohyphal cells that contain $RAS2^{val19}$ are even longer and have even larger axial ratios than CGX19 or CGX31 pseudohyphal cells. Pseudohyphal growth was not observed

for our strains (shr3 or $RAS2^{val19}$) when grown in liquid medium (SPHD).

The Daughter of a Pseudohyphal Cell Can Be a Pseudohyphal Cell or a Blastospore-Like Cell

The elongated pseudohyphal cells have been observed to give rise to either of two cell types. Elongated cells may divide to produce an elongated daughter with roughly the same final dimensions as the mother cell or alternatively a spheroidal blastospore-like cell with roughly the dimensions of a sated yeast cell (Table 2). Blastospores are defined as round or oval budding yeast cells arising from pseudohyphae (Lodder, J. ed., The Yeasts: A Taxonomic Study Amsterdam: North-Holland Publishing Co., 1970). Both the elongate pseudohyphal cell and the blastospore-like cell can be produced either apically or laterally. The blastospore-like cells produced by the pseudohyphal cell may be a new cell type or they may be identical to vegetative cells. When monitored by time lapse photomicroscopy, pseudohyphae are often observed to invade the agar and subsequently begin budding blastospore-like cells at the base of the pseudohypha. These pseudohyphae stop growing altogether and become covered with blastospore-like cells. These blastospore-like cells can divide, showing that at least some of them are actively proliferating.

Pseudohyphal Cells Invade the Semisolid Agar Growth Medium

Pseudohyphal cells penetrate the surface of the agar plate and grow down into the medium. SHR3 diploids as well as other standard strains growing in the sated mode

on rich medium grow by spreading out on the surface of the agar. Even on SPHD medium, most strains grow on the surface. By contrast, SHR3⁻ diploids on SPHD medium are invasive and grow into the agar, presumably in search of nutrients. Columns within the agar have about the same number of members as those on the surface, so the agar represents no deterrent to their exploration. The invasive growth is easily observed in a dissecting microscope and is further demonstrated by the observation that a microneedle must pierce the agar to reach the cells of many pseudohyphae. The mothers and daughters within the chain appear to be physically attached because they often can be manipulated as a unit.

A Mutation in RSR1/BUD1 Causing Random Bud Site Selection Suppresses Pseudohyphal Growth

The growth patterns of strains that budded either in the polar or the random pattern were studied to test the hypothesis that the polar budding pattern of diploids is required for pseudohyphal growth. A dominant mutation of the RSR1/BUD1 gene (Bender and Pringle, Proc. Natl. Acad. Sci. USA 86: 9976-9980 (1989); Ruggieri et al., Mol. Cell Biol. 12: 758-766 (1992)) $rsr1^{asn16}$ (kindly provided by A. Bender) that causes random budding even in the presence of RSR1/BUD1 made it possible to examine the role of budding pattern in pseudohyphal growth. Isogenic diploid strains containing either the $rsr1^{asn16}$ gene on a centromere vector (YCp($rsr1^{asn16}$)) or the vector alone (YCp50) were constructed. The budding pattern of the strains was examined by fluorescence microscopy after staining with Calcofluor (Pringle et al., Meth. Cell Biol. 31: 357-435 (1989)).

Calcofluor stains the chitin in the bud scars and indicates the pattern of previous bud sites on the surface of cells. Both wild type Shr3$^+$ (CGX31) and Shr3$^-$ (CGX19) strains containing the plasmid with the rsr1^{asn16} gene show a random budding pattern whereas the isogenic CGX31 and CGX19 strains carrying the vector alone show the bipolar budding pattern. The consequences of random budding on colony morphology and pseudohyphal growth were examined. The presence of the rsr1^{asn16} allele suppresses pseudohyphal growth of CGX31 and CGX19 on both SPHD (Figure 9) and on low ammonia medium.

The examination of the cells growing at the fringes of the colonies suggests that the random budding pattern caused by rsr1^{asn16} suppresses pseudohyphal growth. At the fringes of the CGX19 rsr1^{asn16} colonies there are a few long cells protruding away from the mass of cells. Their shape, though not as long and thin, resembles that of pseudohyphal Bud$^+$ cells. Their distinguishing feature is that the first daughters of these cells often bud at the middle of their mother rather than her distal tip and grow in a direction perpendicular to the mother's long axis. Thus, despite having an appropriate long cell shape, the disorientation of the daughters prevents cell lineages from developing into pseudohyphal structures which extend beyond the colony margin.

The Dimorphic Transition to Pseudohyphal Growth Permits Foraging for Nutrients

Described herein is pseudohyphal growth, a dimorphic transition in the life cycle of S. cerevisiae. The pseudohypha in S. cerevisiae consists of a lineage of first

daughters associated in a chain. There have been anecdotal references to pseudohyphal growth for this yeast (Guilliermond, A., New York: John Wiley and Sons, Inc., 1920; Brown and Hough, Nature 206:676-678, 1965; Lodder, Amsterdam: North-Holland Publishing Co., 1970; Eubanks and Beuchat, J. Food Sci. 47:1717-1722, 1982 and references in these sources), but no detailed description of the conditions required for its induction. Figure 8 diagrams our current view of the S. cerevisiae life cycle. The radial pattern and invasive character of cell proliferation into the growth substrate clearly is a mechanism that permits cells to forage for nutrients at a distance from their initial position. The unipolar growth pattern manifest by yeast pseudohyphae is the major mechanism by which filamentous fungi proliferate (Rayner, A.D.M., Mycologia 83:48-71, 1991; and references therein).

The Requirements for Pseudohyphal Growth

1. Diploidy and the BUD genes: Only a/α diploids and not a or α haploids or a/a or α/α diploids show pseudohyphal growth, indicating that the mating type locus controls this dimorphic transition. Cells expressing MATa/MATα bud in a polar pattern whereas those expressing only MATa or MATα bud in the axial pattern (Freifelder, D., J. Bacteriol. 80: 567-568 (1960); Hicks, et al., Genetics 85: 395405 (1977); Chant and Herskowitz, Cell 65: 1203-1212 (1991)). The simplest explanation for the control of pseudohyphal growth by the mating type locus is that the polar budding pattern of a/α diploid cells permits linear chains of cells to form; the axial pattern leads to budding at the junction of two cells and cannot

extend the column (Freifelder, D., J. Bacteriol. 80: 567-568 (1960)).

The budding pattern of diploids is controlled by five BUD genes. RSR1/BUD1, BUD2 and BUD5 convert the random pattern into a bipolar pattern and the bipolar pattern is converted into axial by BUD3 and BUD4 (Chant and Herskowitz, Cell 65: 1203-1212 (1991); Chant et al., Cell 65: 1213-1224 (1991); Powers et al., Mol. Cell. Biol. 9: 390-395 (1991)). The polar budding of MATa/α diploids is explained by a1/α2 repression of BUD3 and/or BUD4. Based on this model, it was expected that mutations in RSR1/-BUD1, BUD2 and BUD5 should interfere with the polar cell divisions required for pseudohyphal growth. In agreement with this expectation, a/α cells that bud randomly because of the $rsr1^{asn16}$ ($Bud1^{-}$) mutation are unable to form pseudohyphae. On the basis of these experiments it was reasonable to propose that one role of the BUD genes in yeast biology is to enable cells in the diploid phase to forage for nutrients under conditions of nitrogen starvation.

2. Cell Shape Changes: Pseudohyphal cells are longer and thinner than sated cells growing on rich medium. As cells become longer and thinner, their axial ratio becomes greater and, therefore, the tip of the cell becomes more defined. The ellipsoidal shape of a sated diploid yeast cell confers upon it a low surface area. It is reasonable to surmise that when the growth medium is deficient, the ellipsoidal shape provides insufficient surface area for the cell to extract the diminishing nutrients. A dimorphic transition is induced to adapt to the new environmental conditions and new daughters develop an elongated

morphology. This differentiation creates a shape which increases the surface area and consequently the absorptive surface of the cells.

This change in cell shape has two additional consequences. The first is that the change in cell shape may constrain the plane of cell division along the longitudinal axis of the pseudohyphal cell so that the buds come out very close to the tip (Figure 2B). In a sense the wall of the cell becomes part of the structure orienting the plane of cell division. This is in contrast with polarized growth of sated cells where the buds come out only near to the end, not exactly at the end (Figure 2A). This constraint could occur in much the same way that the shape of the ascus in Neurospora crassa leads to linear asci; in this fungus, the direction of meiotic cell division is confined to the longitudinal plane by the extreme axial ratio of the ascus. The second consequence is that cell growth is polarized along the same axis as cell division. This dual polarity of cell growth and cell division enhances the ability of the growing chain of cells to escape the colony because the growth of each new individual unit of the chain of cells along the axis of cell division incrementally moves the column along. The velocity of pseudohyphal elongation is proportional to the length of the cells which comprise the chain.

3. Unipolar Cell Division: Cell division in the pseudohypha is polarized in one direction, the direction away from the mass of cells in the colony and out into the substrate. This polarization is achieved by four constraints on cell division. First, a terminal pseudohyphal

cell always buds at its free end, the one opposite the junction with its mother. Second, the site of bud emergence on the daughter is close to 180° from that junction. Third, daughters stay juxtaposed to their mothers exactly along the axis of cell division, either because they are physically connected or because they are constrained by the agar. Fourth, the first daughter of the founding mother cell (the cell that gives rise to the column) divides in a direction away from the mass of cells in the colony. This initial division coupled with the three other constraints leads to the polarized extension of pseudohyphae away from the colony into unpopulated substrate.

Sated cells, like pseudohyphal cells, initially bud in a unipolar fashion. The first bud of a virgin stated cell (one that has no prior daughters) as well as the majority of the second buds from these cells emerge from the pole opposite the junction with its mother. If sated diploid cells can bud in a unipolar pattern, it is unclear why they do not form pseudohyphae. First, the buds of sated cells generally separate from their mothers. The detachment of mother and daughter is often associated with movement of the cell that changes the orientation of the daughter with respect to the mother cell. This displacement of the mother-daughter division axes when reiterated in subsequent divisions ultimately leads to randomization of the division planes of an individual mother with respect to any founding cell or starting point. Second, although the buds on sated cells arise on the surface opposite the mother-daughter junction, the position of the new bud is not always 180° from that junction. The sated

cells are ellipsoidal so the poles are less defined than in the elongate pseudohyphal cells. Any changes in the angle of the bud with respect to the axis of the previous mother-daughter junction contribute to unpolarized growth. An extreme example of the effect of bud angle is demonstrated by the $rsr1^{asn16}$ mutation which, by randomizing the sites of bud formation, prevents diploids from forming pseudohyphae.

Previous workers have noted that sated mother cells that have divided at least once (non-virgin) show bipolar cell division (Freifelder, D., J. Bacteriol. 80: 567-568 (1960); Chant and Herskowitz, Cell 65: 1203-1212 (1991)). In bipolar cell division buds can emerge on either the birth or free ends of mother cells that are not virgin. It is not known how many divisions must occur before the unipolar pattern is replaced by the bipolar pattern. Since the cell at the tip of the pseudohypha is always a virgin cell, this apical cell always buds in a unipolar fashion. When cells in the pseudohypha bud for a second time, they bud in a unipolar pattern about 90% of the time. When sated cells bud for a second time, they also generally bud unipolarly although about 20% less frequently than pseudohyphal cells. This phenomenon suggests that nutritional factors may regulate the budding pattern of diploid cells.

4. Invasiveness: Pseudohyphal cells are invasive and grow into the agar, presumably foraging for nutrients. Some of the foraging columns of cells have as many as 10 members in the main chain. Columns within the agar have about the same number of cells as those on the surface, so

the agar is no deterrent to their exploration. The invasiveness of pseudohyphal cells can be explained in several ways. One might imagine that the force of unipolar cell division by thin pseudohyphal cells is sufficient to propel a column through the agar. In the pseudohyphal cycle, cell separation, a late step in cell division, may be delayed, leaving the daughters attached to the mother. This linked structure might be able to generate more force than a single cell because the previous generations could act as an anchor for the cell at the apex. Although this mechanical model may be correct, the connection between the apical cell in the pseudohyphal column and its mother can sometimes be broken by mechanical agitation with a microneedle. Furthermore, flocculent strains and cell-cycle mutants defective in cell separation do not grow invasively, showing that cell separation defects alone do not cause invasiveness.

The secretion of hydrolytic enzymes is likely to be an important factor permitting invasive growth. Lytic enzymes capable of hydrolysing polysaccharides may be secreted by strains capable of pseudohyphal growth. The secretion of proteases is common to many invading pathogens such as Candida albicans (Macdonald and Odds, J. Gen. Microbiol. 129: 431-438 (1983)). Hydrolytic enzymes in C. albicans are important in creating a pathway for penetration into the host tissue. By analogy, the invasive habit of pseudohyphal S. cerevisiae cells may be a growth pattern used in nature to penetrate natural substrates.

Conclusions

The results described herein are consistent with the following model: the SHR3 gene product is required for the proper sorting of amino acid permeases in the ER. Mutations in SHR3 which abolish its ability to interact specifically with amino acid permeases block the entry of amino acid permeases into the secretory pathway. Membrane proteins, including amino acid permeases, targeted to the plasma membrane or intracellular organelles, e.g., vacuole, are dependent upon the secretory pathway for transit to their proper destinations. Consistent with this model we have observed the accumulation of GAP1 in the endoplasmic reticulum. If SHR3 is involved in sorting it must specifically interact with amino acid permeases since other proteins destined to the plasma membrane are not affected by shr3 mutations. It should be noted that the yeast amino acid permeases exhibit a high degree of sequence homology (four permease genes have been cloned). It is therefore not reasonable to imagine that SHR3 interacts with a particular domain shared by these amino acid permeases. Further genetic analysis will be necessary to define the protein-protein interactions required for proper sorting.

Implications of Dimorphic Growth and Diploidy for Pathogenesis

The interconversion of a yeast form and a filamentous form is typical of many pathogenic fungi (Shepherd, M.G., Curr. Top. Med. Mycol. 2: 278-304 (1988)). In C. albicans, a human pathogen, the ability to undergo a dimorphic transition is critical for pathogenesis (Soll, D.R., New

York, New York: Plenum Press, 503-540 (1991)). C. albicans, the causative agent of vaginal candidiasis and often fatal systemic infections in immunocompromised hosts, is found only as a diploid; no sexual cycle has been observed (Odds, F.C. ed., London: Bailliere Tindal (1988)). In tissue infected with Candida, yeast cells, pseudohyphae and true hyphae are found (Odds, F.C. ed., London: Bailliere Tindal, 1988). This conversion to pseudohyphal growth in Candida as in Saccharomyces involves a switch in cell division that requires the unipolar budding pattern displayed by both diploid S. cerevisiae cells (Freifelder, D., J. Bacteriol. 80: 567-568 (1960) and C. albicans cells (Chaffin, W.L., J. Gen. Microbiol. 130: 431-440 (1984)). The requirement of unipolar diploid budding for pseudohyphal growth could explain why Candida is found only as a diploid (no haploid form or sexual cycle has been observed, Odds, F.C., ed., London: Bailliere Tindal, 1988). Perhaps Candida once had a diploid phase, but with time and selection, genes required for meiosis and therefore the generation of haploid cells were lost.

Ustilago maydis, the causative agent of corn smut, is pathogenic only in its filamentous form. The haploid phase of this fungus grows exclusively in a yeast form and is nonpathogenic (Schulz et al., Cell 60: 295-306 (1990)). Although Saccharomyes, an ascomycete, and Ustilago, a basidiomycete, are quite distant on a phylogenetic scale, the major morphogenetic event in each species, conversion of the yeast to a filamentous form, has similar genetic control. Both Saccharomyces and Ustilago haploids grow as yeast cells unable to develop into their filamentous form. Saccharomyces diploids and Ustilago dikaryons heterozygous

for mating type loci undergo a dimorphic transition to filamentous growth. In Saccharomyces diploids (MATa/-MATα), the heterodimeric repressor a1α2 (Goutte and Johnson, Cell 52: 875-882 (1988); reviewed by Herskowitz, I., Nature 342: 749-757 (1989) encoded by the mating type loci must be required for the conversion because isogenic MATα/MATα or MATa/MATa strains do not undergo the dimorphic transition. We surmise that a1α2 repression of BUD3 and/or BUD4, two genes that convert the diploid to the haploid budding pattern, is required for conversion to the pseudohyphal form. In Ustilago where the mating type loci are also thought to encode transcription factors (Schulz et al., Cell 60: 295-306 (1990), heterozygosity at the mating type loci may also be required to repress haploid specific cell division patterns because dikaryons homozygous at the mating type loci cannot undergo the dimorphic transition and are nonpathogenic.

Because of the relationship between the dimorphic transition and pathogenicity, genes which affect the dimorphic switch, such as SHR3, as well as the mRNA and proteins encoded by such genes, may be targeted by antifungal agents. Because overexpression of SHR3 inhibits pseudophyphal growth, administration of plasmids containing the SHR3 gene; of an mRNA of the gene; or of the protein encoded by the gene, can be used to maintain a fungus in the non-pathogenic form. Genes homologous to SHR3, as well as their mRNA and encoded proteins, can similarly be used. In addition, agents which enhance expression of the SHR3 gene or of other comparable genes in other fungi can be administered to suppress pseudohyphal (pathogenic) growth of the fungus; agents

which block inhibitors of SHR3 and its protein or of other comparable genes and proteins can be administered to ensure the suppression of pseudohyphal growth.

While a fungus is thus maintained in the non-pathogenic state, an additional agent to kill the fungus can be administered. This antifungal agent can be administered in conjunction with or sequentially to the agent used to maintain the fungus in the non-pathogenic state. Agents can be administered intravenously, topically, orally, rectally, nasally, buccally, vaginally, or by inhalation spray. The method by which the agents are administered depends in part on the location of the fungal infection; the form in which the agents are administered (e.g., capsule, tablet, solution, emulsion), depends in part on the route of administration.

The procedures and experiments used in the work described herein are illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLES

EXAMPLE 1 Experimental Procedures

A. Media and Microbiological Techniques

Standard yeast media were prepared and yeast genetic manipulations were performed as described in Sherman et al., Methods in Yeast Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986). Departures from standard media are all variations of SPHD (synthetic

proline histidine dextrose) medium that contains 6.7 g/l Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco Laboratories), 1.0 g/l L-proline as sole nitrogen source (Sigma Grade from Sigma), 2% anhydrous D-glucose (from J.T. Baker), and 0.2 mM L-histidine hydrochloride (from Sigma). In SAHD (synthetic ammonia histidine dextrose) and SRHD the proline was replaced with 1.0 g/l of ammonium sulfate (from J.T. Baker) 1.0 g/l of L-arginine (from Sigma) respectively. SPAHD and SPRHD media contain respectively 0.5 g/l L-proline +0.5 g/l ammonium sulfate or 0.5 g/l L-proline + 0.5 g/l L-arginine as sole nitrogen sources. SLAHD (low ammonia) contains only 0.05 mM ammonium sulfate as sole nitrogen source and is made with washed agar.

SPD is a non-standard synthetic medium that contains 6.7 grams/liter Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco Laboratories), 1.0 gram/liter L-proline as sole nitrogen source, 20 grams/liter D-glucose. Where required, SPD was supplemented with either 1 mM or 30 mM L-histidine; appropriate volumes of a filter sterilized 0.5 M L-histidine stock soution were added and the pH was adjusted to 5.5 with 10 N NaOH. The concentration of Yeast Nitrogen Base in SPD is four fold higher than the amount used in other standard synthetic media because this amount was found to enhance the toxicity of histidine and reduced background growth of wild-type strains durng shr mutant screens. SUD medium is the same as SPD except that 1.0 gram/liter urea is substituted for proline as the sole nitrogen source. Solid SPD and SUD media were prepared as follows. The nitrogen sources (4 grams/liter) and the Yeast Nitrogen Base (26.8

grams/liter) were combined to make 4X stock solutions that were filtered sterilized. Other components were autoclaved as separate stock solutions (40% glucose adn 4% Difco Bacto agar). Stock solutions and sterile water were mixed to make a 2X solution, and an equal volume of molten 4% agar was added.

Standard 100 x 15 mm plastic petri dishes were filled with 25 ml of medium. These plates yielded uniform and consistent results only when used during the first week following preparation. This period could be lengthened to 2-3 weeks by washing the agar a few times with water before autoclaving. Yeast transformations were performed by the lithium acetate method of Ito et al., J. Bacteriol. 153: 163-168 (1983) using 30μg to 50μg of sonicated or heat denatured calf thymus DNA as carrier. Transformants were selected on solid SC media lacking appropriate auxotrophic supplements.

Total yeast protein was obtained by the method of Silve et al. MOl. Cell. Biol. 11: 1114-1124 (1991). Samples were heated for 10 min at 37°C and proteins were resolved by SDS-PAGE using a modified Laemmli system (Laemmli, Nature 227: 680-985 (1970)) in which SDS is omitted from the gel and lower electrode buffer. Endoglycosidase H treatment was carried out according to Orlean et al., (1991). Immunoblots were processed as described by Kim et al. Methods Enzymol. 194: 682-697 (1990)). Primary antibodies were used at the following dilutions: anti-HA1 mouse monoclonal 12CA5 culture supernatants, 1:50; guinea pig anti-invertase antisera (the kind gift of Daphne Preuss), 1:2500; anti-PMA1 mouse monoclonal F10-9 ascites fluid (kindly provided by John

Teem), 1:1000; affinity purified polyclonal rabbit anti--PMA1 antiserum (kindly provided by Amy Chang), 1:2000. Blots probed with primary rabbit or guinea pig antibodies were incubated 1-2 hr with affinity purified [^{125}I]-Protein A (100 μCi/ml, Amersham Corporation) diluted 1:2000. Blots probed with primary mouse antibodies were incubated 1-2 hr with affinity purified rabbit anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:500, washed, and then incubated with protein [^{125}I]-Protein A diluted 1:-2000.

Yeast growth in low sulfate synthetic medium, invertase induction, and pulse labeling were carried out; immunoprecipitation of carboxypeptidase Y and alpha-factor was so carried out; and analysis of electrophoretically resolved ^{35}S-labeled proteins, gels fixing preparation for fluorography, and exposure to film are all described in Rothblatt and Schekman. (Methods Cell Biol. 32: 3-36 (1989)).

Membranes were prepared from cells grown in SUD (+ adenine and uracil) essentially as described by Chang and Slayman J. Cell. Biol. 115: 289-295 (1991)). Cells were grown to an OD600nm of 1.5, harvested by centrifugation, washed once in BB buffer (10 mM Tris pH 7.5, 5 mM $MgCl^2$, 0.1 M NaCl, 0.3 M sorbitol), and resuspended in BB buffer at 200 OD_{600nm} units ml^{-1}. Protease inhibitors were added and cells were lysed by vortexing with glass beads (3 X 1 min pulses). The cell lysate was centrifuged at 400 g for 5 min to remove unbroken cells, and a total membrane fraction was obtained by centrifugation at 100,000 g for 1 hr. Pelleted membranes were resuspended in a minimal volume of M buffer (20 mM HEPES [N-2-hydroxyethyl-piperazine-N'-2-

ethanesulfonic acid], pH 7.4, 250 mM sucrose) at an average protein concentration of 36 mg ml^{-1}, subdivided into small aliquots, and stored frozen at -70°C. Membrane protein was determined by the method of Markwell et al. (1978).

GAP1 membrane association was determined as described by Deshaies and Schekman Mol. Cell. Biol. 10: 6024-6035 (1990)). Fifty micrograms of membrane protein was diluted into 80 µl of M buffer. Twenty microliters of either M buffer, 2.5% Triton X-100, 0.5 M Na_2CO_3 (pH11), 8 M urea, or 3 M NaCl were added, samples were incubated at 4°C for 15 min, and centrifuged at 100,000 g for 1 hr. The resulting pellets were resuspended in 50 µl SDS-PAGE sample buffer and heated at 55°C for 10 min. Twenty microliter aliquots were resolved by SDS-PAGE, and immunoblots were analyzed using the monoclonal antibody 12CA5 as previously described. Blots were incubated 1-2 hr with affinity purified rabbit anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:500, washed, and then incubated with protein [^{125}I]-Protein A diluted 1:2000. The amount of radioactivity was quantitated using a Fujix Bio-Image Analyzer BAS2000 (Fuji Photo Fim Co., Ltd., Japan).

GAP1 protease sensitivity was examined by limited trypsin digestion. Fifty micrograms of membrane protein suspended in 50 µl of M buffer were digested with varying trypsin concentrations for 90 min at 4°C. After digestion was terminated by the addition of 2 µl freshly prepared 0.1 M PMSF, the samples were incubated an additional 10 min at 4°C. Twenty microliters of 5X SDS-PAGE sample buffer was added and samples were heated at 55°C for 10 min. Thirty-five microliter aliquots were resolved by

SDS-PAGE, and the resulting immunoblots were analyzed using the monoclonal antibody 12CA5 as previously described. GAP1 was visualized either with [^{125}I]-Protein A as previously described or with chemiluminescence detection reagents (ECL Western Blotting Detection System, Amersham International).

B. Yeast Strain Construction

Yeast strains are listed in Table 3. Several different mutant alleles of SHR3 gave rise to enhanced pseudohyphal growth on SPHD. These include both an in vitro constructed null allele SHR3Δ1::URA3 (Ljundahl et al., in preparation) and a spontaneously isolated allele SHR3-102. Each of these when homozygous in a MATa/α diploid gives rise to pseudohyphal growth. As indicated in the Results section, strains from the Σ1278b background give the most extensive pseudohyphae. Therefore, experiments were carried out in this background. S. cerevisiae strains MB1000 (MATΣ, Brandriss and Magasanik, J. Bacteriol. 143: 1403-1410 (1979)) and MB758-5B (MATa ura3-52, Siddiqui and Brandriss, Mol. Cell. Biol. 8: 4634-4641 (1988)) were obtained from M. Brandriss. MB1000 is also known in the literature as Σ1278b (Grenson et al., Biochim. Biophys. Acta. 127: 325-338 (1966)). The ura3-52 mutation in MB7S8-5B originates from strain DBY785 and was introduced by a cross with MB1000. A ura3-52 segregant from this cross was made congenic to Σ1278b by performing 10 backcrosses to MB1000 resulting in MB758-5B (Siddiqui and Brandriss, Mol. Cell. Biol. 8: 4634-4641 (1988)). PLY4 was constructed from PLY1; the mating type was switched by transformation with plasmid pGAL-HO (Herskowik and Jensen,

Methods Enzymol. 194: 132-146 (1991)), and the ADE2 gene was deleted and replaced with the selectable marker URA3 by transformation with BamHI digested plasmid pPL132. F35 (MATa/α HO/HO apf/apf) is a spontaneous diploid derivative of strain RA68 (MATα apf) originally obtained from M. Grenson. F35 was sporulated and mated with MB758-5B (MATa ho ura3-52). The resulting diploid CGDY53 (MATa/α HO/ho apf/APF ura3-52/URA3) was sporulated, and a stable mating segregant CGAS53-2E (MATa, ho apf ura3-52) was obtained.

A ura3-52 shr3⁻ mutant strain in the Σ1278b background was produced by obtaining a spontaneous mutant of MB758-5B resistant to 30 mM histidine. These conditions allow the positive selection of shr3⁻ mutants. The particular allele we chose (shr3-102 in CG25) was shown to be an allele of SHR3 by the following tests: 1) it was recessive to SHR3 and failed to complement the 30 mM histidine growth or the enhanced pseudohyphal growth of a known loss of function shr3⁻ allele; 2) it was complemented for both the growth at 30 mM histidine and enhanced pseudohyphal growth phenotypes by a plasmid containing a 1.4 kb genomic fragment (pPL210) that contained only the SHR3 coding region, and 3) when it was crossed by an SHR3 strain, the 30 mM histidine growth phenotype segregated in a Mendelian fashion in tetrads. We called this mutant allele shr3-102.

CG25 (MATa ura3-52 shr3-102) was backcrossed to MB1000 (MATα) and segregants with the following genotypes were identified and isolated: MATα ura3-52 shr3-102 (CG41), MATa ura3-52 (CG46), MATα ura3-52 (CG48). A MATa/α ura3-52/ura3-52 shr3102/shr3-102 diploid (CGX19) was constructed by crossing CG25 x CG41. A MATa/α ura3--

52/ura3-52 diploid (CGX31) was constructed by crossing CG46 x CG48. Spontaneous MATa/a and MATα/α ura3-52/ura3-52 shr3-102/shr3-102 derivatives of CGX19, CG85 and CG67 respectively, were isolated by obtaining spontaneous mitotic recombinants homozygous at the MAT locus from CGX19 cells. Isogenic pairs of diploid strains that differ only in their SHR3, RAS2, or RSR1/BUD1 alleles were produced by transformation with centromere plasmids and selection of transformants on solid SC-uracil medium (see Table 3).

TABLE 3

Saccharomyces cerevisiae Strains

Strain	Genotype	Source or Reference Comments
MB1000	MATα	Brandriss and Magasanik(1979)
MB758-5B	MATa ura3-52	Siddiqui and Brandriss (1988)
CG25	MATa ura3-52 shr3-102	This work, MB758-5B derivative
CGX15	MATa/α ura3-52/URA3 shr3-102/SHR3	Gimeno et. al (1992)
CG41	MATα ura3-52 shr3-102	Ascospore from CGX15
CG46	MATa ura3-52	Ascospore from CGX15
CG48	MATα ura3-52	Ascospore from CGX15
CGX19	MATa/α ura3-52/ura3-52 shr3-102/shr3-102	CG25 x CG41
CGX31	MATa/α ura3-52/ura3-52	CG46 x CG48
CG67	MATα/α ura3-52/ura3-52 shr3-102/shr3-102	MATα/α derivative of CGX19
CG85	MATa/a ura3-52/ura3-52 shr3-102/shr3-102	MATa/a derivative of CGX19
CG62	MATa/α ura3-52/ura3-52 shr3-102/shr3-102 (pPL210)	CGX19 + pPL210
CG64	MATa/α ura3-52/ura3-52 shr3-102/shr3-102 (pRS306)	CGX19 + pRS306
CGX69	MATa/α ura3-52/ura3-52 (YCpR2V)	CGX31 + YCpR2V
CGX71	MATa/α ura3-52/ura3-52 (YCp50)	CGX31 + YCp50
CGX73	MATa ura3-52 (YCpR2V)	CG46 + YCpR2V
CGX75	MATα ura3-52 (YCpR2V)	CG48 + YCpR2V
CG132	MATa/α ura3-51/ura3-52 (YCp($rsr1^{sen16}$))	CGX31 + (YCp($rsr1^{sen16}$))
CG133	MATa/α ura3-52/ura3-52 shr3-102/shr3-102 (YCp50)	CGX19 + YCp50

CG134	MATa/α ura3-52/ura3-52 shr3-102/shr3-102 (YCp(rsr1^{asn16}))	CGX19 + YCp(rsr1^{asn16})
RA68	MATα apf	Grenson and Hennaut, 1971
F35	MATa/α HO/HO apf(shr3-101J/apf(shr3-101)[b]	This work
MB758-5B	MATa ho ura3-52	Siddiqui and Brandriss, 1988
CGDY53	MATa/α HO/ho apf(shr3-101)/APF(SHR3) ura3-52/URA3	This work
CGAS53-2E	MATa ho apf(shr3-101) ura3-52	This work

Isogenic Derivatives of PLY1

PLY1	MATa his4Δ29 ura3-52	This work
PLY4	MATα his4Δa29 ura3-52 ade2Δ1::URA3	This work
PLAS1-7D	MATa ura3-52 his4Δ29	This work
PLAS3-4A	MATa shr3-3 ura3-52 his4Δ29	This work
PLAS16-6B	MATα shr3-16 ade2Δ1::URA3 ura3-52 his4Δ29	This work
PLAS16-6C	MATα SHR3 ade2Δ1::URA3 ura3-52 his4Δ29	This work
PLAS23-4B	MATα shr3-23 ura3-52 his4Δ29	This work

Isogenic Derivatives of AA280

AA280	MATα ura3-52 his3Δ200 lys2Δ201 ade2	Antebiand Fink, 1 992
AA288	MATa ura3-52 leu2-3,112 lys2Δ201 ade2	Antebiand Fink, 1 992
AA305	MATa/MATα HIS3/his3Δ200 LEU2/leu2-3,112 ura3-52/ura3-52 lys2Δ201/lys2Δ201 ade2/ade2	Antebiand Fink, 1992
PLY129	MATa ura3-52 leu2-3,112 lys2Δ201 ade2 gap1α::LEU2	This work
PLY134	MATa ura3-52 leu2-3,112 lys2Δ201 ade2 gap1Δ::LEU2 shr3Δ1::URA3	This work
PLY143	MATa ura3-52 lys2Δ201 ade2	This work
PLY145	MATa ura3-52 lys2Δ201 ade2	This work
PLY148	MATα ura3-52 lys2Δ201 ade2	This work
PLY152	MATa ura3-52 lys2Δ201 ade2 shr3Δ1::URA3	This work
PLY158	MATα ura3-52 lys2Δ201 ade2 shr3Δ1::URA3	This work

10053-3A	MATa cdc16-1 his4-619 ura3-52	Fink lab collection
CGX56	MATa/α trp1::hisG-URA3-hisG/Trp1 ura3-52/URA3	CG112 X MB1000
CGX66	MATa/α ura3-52/URA3	Gimeno et al., (in press)
CGX73	MATa/α trp1::hisG/TRP1 ura3-52/URA3	CG182 X MB1000
CGX80	MATa/α phd1Δ1::URA3/PHD1 trp1::hisG/TRP1 ura3-52/ura3-52	CG238 X CG188
CGX86	MATa/α phd1Δ1::URA3/PHD1 cdc16-1/CDC16 trp1::hisG/TRP1 his4-619/HIS4 ura3-52/ura3-52	10053-3A X CG245
CGX93	MATa/α phd1Δ1::URA3/PHD1 cdc16-1/CDC16	CG290 X CG289

	trp1::hisG/TRP1 his4-619/HIS4 ura3-52/ura3-52	
CGX94	MATa/α phd1Δ1::URA3/PHD1 cdc16-1/CDC16 trp1::hisG/TRP1 his4-619/HIS4 ura3-52/ura3-52	CG343 X CG344
CG112	MATa trp1::hisG-URA-3-hisG ura3-52	CG46 transformed with BgIII-EcoRI digested pNKY1009
CG139	MATa trp1::hisG-URA3-hisG ura3-52	Ascospore from CGX56
CG145	MATa ura3-52	Ascospore from CGX66
CG146	MATa ura3-52	Ascospore from CGX66
CG151	Mata/α ura3-52/ura3-52 (PCG7)	CGX69 + pCG7
CG157	MATa/α ura3-52/ura3-52 (pRS202)	CGX69 + pRS202
CG182	MATa trp1::hisG ura3-52	5-FOA segregant from CG139
CG188	MATα trp1::hisG ura3-52	Ascospore from CGX73
CG234	MATa/α ura3-52/ura3-52 (pCG38)	CGX69 + PCG38
CG238	MATa phd1Δ1::URA3 ura3-52	MB758-5B transformed with SalI-SacI digested pCG36
CG245	MATα phd 1Δ1::URA3 trp1::hisG ura3-52	Ascospore from CGX80
CG289	MATα cdc16-1 ura3-52	Ascospore from CGX86
CG290	MATa phd1Δ1::URA3 his4-619 trp1::hisG ura3-52	Ascospore from CGX86
CG343	MATa phd1Δ1::URA3 cdc16-1 trp1::hisG ura3-52	Ascospore from CGX93
CG344	MATα his4-619 ura3-52	Ascospore from CGX93

Plasmids

Strain	Genotype	Source or Reference Comments
pPL210	1.4kb fragment containing SHR3 in pRS306	
pRS316	URA3 marked centromere vector	Sikorski and Hieter (1989)
YCpR2V	$RAS2^{val19}$ in YCp50	$RAS2^{val19}$ is described in Kataoka et al. plasmid M. obtained from Wigler.
YCp ($rsr1^{asn16}$)	$rsr1^{asn16}$ in YCp50	Ruggieri et al. (1992) Obtained from Bender). A.
pPL130	6.2 kb fragment containing ADE2 in pUC19	This work
pPL132	ade2Δ1::URA3 inpUC19	This work
pPL152	9.6 kb fragment containing SHR3 in YCp50	This work
pPL153	9.6 kb fragment containing SHR3 in YCp50 (pPL153 appears to be identical to pPL152)	This work
pPL154	13 kb fragment containing SHR3 in YCp50	This work
pPL155	12 kb fragment containing SHR3 in YCp50	This work
pPL164	11 kb BamHI fragment containing SHR3 in pRS316	This work
pPL179	3 kb Kpn1-EcoRI fragment containing SHR3	This work

	in pRS31 6	
pPL183	4 kb EcoRI-BamHI fragment containing SHR3 in pRS31 6	This work
pPL202	1.4 kb Acci fragment containing SHR3 in pBSII SK(+)	This work
pPL210	1.4 kb AccI fragment containing SHR3 in pRS316	This work
pPL216	shr3Δ3 in pBSII SK(+)	This work
pPL219	shr3Δ1::URA3 in pBSII SK(+)	This work
pPL230	SHR3::FLU2 in pRS316	This work
pPL247	3.5 kb Sa/l-SpeI fragment containing GAP1 in pRS316	This work
pPL257	GAP1::FLU1 in pRS316	This work
pPL258	GAP1::FLU2in pRS316	This work
pPL262	3.5 kb Sa/l-SpeI fragmenl containing GAP1 in YCp405	This work
pPL269	GAP1::FLU1 in YCp405	This work
pPL289	GAP1::FLU2inYCp405	This work
pGAL-HO	HO gene under control of GAL10 promoter	Herskowik and Jensen 1 991
pMS16	6.0 kb BamHI-Sa/1 fragment containing GAP1 in pBS KS(+)	M. Stanbrough (unpublished data)
pMS20	GAP1Δ::LEU2 in pBS KS(+)	M. Stanbrough (unpublished data)
p180	GCN4-LacZ in YCp50 (regulated expression)	Hinnebusch, 1985
p227	gcn4-LacZ in YCp50 (constitutive expression)	Mueller and Hinnebusch, 1986
pCG31	3.1 kb pCG7 fragment with PHD1 in pBSIIKS+	This work
pCG34	2.0 kb fragment with phd1Δ1 in pBSIIKS+	This work
pCG35	3.1 kb fragment with PHD1::FLU1 in pBSIIKS+	This work
pCG36	7.0 kb fragment with phd1Δ::URA3 in pBSIIKS+	This work
pCG37	2.6 kb fragment with PHD1::FLU1 in pRS202	This work
pCG38	2.6 kb fragment (BgIII-HindIII) with PHD1 in pRS202	This work
pCG40	2.6 kb fragment with PHD1 in pRS316	This work
pCG41	2.6 kb fragment with PHD1 in pRS305-2μ	This work
pBSIIKS+	Escherichia coli vector	(Stratagene)
pNKY1009	TRP1 insertion vector	Alani et al., (1987)
pR305-2μ	LEU2 marked 2μ vector	Miller and Fink, (unpublished data)
pR316	URA3 marked CEN vector	Siorski and Hieter, (1989)
pRS202	URA3 marked 2μ vector	Connelly and Hieter, (unpublished data)
pSE1076	5.0 kb hisG-URA3-kan'-hisg fragment in vector	Elledge, (unpublished data)

[a]All strains with the exception of CGX86, CGX93, CCGX94, 10053-3A, CG245, CG290, CG289, CG343, and CG344 are congenic to the Σ1278b genetic backround (Grenson et al., 1966).

[b]The apf allele of F35 was renamed shr3-101 to conform with standardized yeast genetic nomenclature.

c. Bud Site Selection Assay

Photomicrographs of developing pseudohyphae in colonies of CGX19 (a/α shr3⁻) that had been growing for 1 to 3 days on SPHD + uracil medium at 30°C were taken at time intervals. All lineages in which the origin of the terminal 3 cells could be determined by either time lapse observation were used. Emergence of the first bud of the virgin mother's first daughter was scored as from the free or birth end. Following the conventions of Freifelder (J. Bacteriol. 80: 567-568 (1960)), the pole of a bud which contacts its mother cell is called the birth end and the opposite pole the free end. Emergence of the virgin mother's second bud after the time required for two cell divisions was scored as either occurring at the free end or not occurring at the free end. There were 11 instances where no second bud emerged on the free end of the mother cell. In each of these cases the birth end was obscured by neighboring cells so the presence of a bud at the birth pole could not be scored. These 11 events were tabulated as birth end buds. This scoring strategy was used because it allowed the incorporation of all cell divisions visible by time lapse photomicroscopy into the data set.

The budding pattern of sated virgin CGX19 cells was analyzed by patching out CGX19 on YPD medium, supplemented with 20 mg/l of adenine sulfate, pregrowing the cells for 2 days at 30°C, and then micromanipulating cells with small buds onto a YPD plate in a grid pattern. After 7 to 9 hours of growth at 24°C all cells except for virgin cells with a small bud (one per cell originally placed on plates if this cell grew normally) were micromanipulated away from the grid. The positions of the virgin cell and its first bud were recorded at the beginning of the exper-

iment and at time intervals. All virgin cells that gave rise to microcolonies of 4 cells within a 6 hour period at 24°C were scored. Emergence of the first bud produced by the original virgin cell's first daughter was scored as emerging from its free or birth end. Emergence of the virgin mother's second bud was scored as either from the free or birth end, assuming that the pole from which the first bud emerged is the free end as discussed.

D. Microscopy

1. Scanning Electron Microscopy

Yeast cells proliferating on agar growth medium were transferred with a toothpick to small squares of wet Schleicher and Schuell #576 filter paper. The cells on the paper were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) at 24°C for 60 min and dehydrated in a graded ethanol series at 24°C. This material was critical point dried in liquid carbon dioxide, mounted on SEM stubs, and then sputter coated with gold and palladium. SEM was performed on the upper stage of an ISI-DS130 scanning electron microscope and the images were photographed on Polaroid 55 film.

2. Light Microscopy

Light microscopy of single cells and microcolonies was done with a Zeiss WL light microscope using bright field optics. Petri plates were placed directly on the microscope stage. A 40X short working distance objective and 32X and 2.5X long working distance objectives, all from Zeiss, were used to visualize cells or colonies. Some light microscopy of macrocolonies was done with a Wild M5A stereomicroscope with a transmitted light console

base. Light photomicroscopy for quantitation of single cell dimensions was done with a Zeiss Axioskop using Nomarski optics. Either 40X (for pseudo-hyphae) or 100X (for single cells) objectives were used.

3. Fluorescence Microscopy

Staining of fixed yeast cells by indirect immunofluorescence was carried out essentially as described by Davis and Fink (1990). Cells transformed with epitope tagged plasmid constructions were pre-grown to a density of 1 x 10^7 cells ml^{-1} in SC - ura or SUD in order to select for plasmid maintenance. Cells were then diluted to a density of 2 x 10^6 cells ml^{-1} in either YPD or fresh SUD media, as indicated and grown for an additional 5 hr. Freshly prepared 40% formaldehyde was added directly to the cells in growth medium to a final concentration of 4%. Cells were fixed overnight on ice, washed twice in solution B (0.1 M potassium phosphate buffer, pH 7.5 in 1.2 M sorbitol) and resuspended to 1 x 10^8 cells ml^{-1} in solution B containing 30 μM β-mercaptoethanol. Oxalolyticase (Enzogenetics, Corvallis, OR) was added to a final concentration of 0.1 mg ml^{-1}, and cells were incubated at 30°C. Spheroplasting was stopped by dilution of cells into 15 ml of ice cold solution B. Spheroplasts were collected by centrifugation, resuspended to 1 x 10^8 cells ml^{-1} in solution B, and pipetted onto polylysinecoated round coverslips. After 30 min the cell suspension was gently aspirated away, cover slips were covered with incubation buffer (solution B containing 4% instant milk) and incubated an additional 15 min. The cover slips were washed twice with solution B, covered with 100% methanol (incubated for 5 min at -20°C) and washed three more times with

solution B. The cover slips were incubated in incubation buffer for 2 hr at 30°C. Primary antisera incubations were done at 30°C for 2 hr in incubation buffer (see figure legends for dilutions). Subsequent washes, secondary antibody incubations, DAPI staining, microscopy and photography were carried out as described (Davis and Fink, 1990). The secondary antibodies were affinity purified fluorescent DTAF-conjugated antibodies (Jackson Labs, West Grove, PA; DTAF: 5-[(4,6-dichlorotriazin-2-y) amino-]fluorescein) diluted 1:50 in incubation buffer. Either DTAF-conjugated goat anti-mouse IgG or DTAF-conjugated donkey anti-rabbit IgG were used.

E. Quantitation of Yeast Cell Dimensions

Measurements of pseudohyphal and sated cell dimensions were based on photomicrographs of cells from colonies obtained by streaking CGX19 (MATa/α ura3-52/ura3-52 shr3-102/shr3-102) for single cells on agar plates. Sated and pseudohyphal cells used for quantitation by SEM were, respectively, from YPD and SPHD + uracil plates incubated at 30°C for 31 hours. The pseudohyphal cells and blastospore-like cells used for light photomicroscopic quantitation were from the same SPHD + uracil plate incubated at 300°C for 7 days. Blocks of agar 1.0 cm x 0.5 cm containing several polarized colonies were lifted from the plate with a scalpel. A thin piece of agar from the surface 0.2 to 0.3 cm thick containing the colonies and their associated invasive pseudohyphae was removed from the block, transferred to a slide, and a cover slip was applied to it without pressure. Photomicrographs of invasive pseudohyphae and their associated blastospore-like cells were made with a 40X objective with Nomarski optics. The sated

cells used for light photomicrographic quantitation were from YPD plates incubated at 30°C for 26 hours. To reproduce the optical conditions of the invasive pseudohyphae and blastospore-like cells, sated cells from a colony on the agar surface were suspended in a 37°C solution of 0.6 M sorbitol, 5% glucose and 1% low melting point agarose and dropped onto a slide. A coverslip was quickly applied and pressed down to form a thin layer of agar-suspended cells. Photomicrographs were taken after the agar had solidified with a 100X oil immersion objective with Nomarski optics. All measurements were converted to μm.

EXAMPLE 2 Genetic, Physiological and Morphological Characterization of S. Cerevisiae Pseudohyphal Growth

A. Physiological Characterization of PseudohyphAl Growth

MATa/α ura3-52/ura3-52 (CGX31) and MATa/α ura3-52/-ura3-52 shr3-102/shr3-102 (CGX19) were streaked for single cells on SLAHD plus uracil, SPHD plus uracil, or SAHD plus uracil plates, incubated at 30°C for 48 hr, and the resulting colonies were photographed. Results are shown in Figure 9. (A), (B), (C) and (D) show low magnification views of colonies of: (A) strain CGX31 growing on SLAHD plus uracil, (B) CGX31 growing on SPHD plus uracil, (C) CGX19 growing on SPHD plus uracil, and (D) CGX19 growing on SAHD plus uracil. In (B) the three colonies with pseudohyphae are designated with arrows. (E), (F), (G) and (H) show high magnification views of the colonies marked by large arrows in (A), (B), (C) and (D). (A), (B), (C) and (D) have the same scale, with the scale bar in (C) representing 0.5 mm (E), (F) and (G) have the same scale,

with the scale bar in (F) representing 30 μm. The scale bar in (F) represents 30 μm.

B. SEM Analysis of Starvation-Induced CelL Morphology Changes

CGX19 MATa/a ura3-52 shr3-102/shr3-102 growing vegetatively at 30°C was streaked for single colonies on prewarmed (A) YPD and (B) SPHD plus uracil plates. These plates were incubated at 300°C for 31 hr and then prepared for SEM as described in Experimental Procedures. Results are shown in Figure 2. (A) shows a representative YPD--grown sated yeast cell that has been budding in a bipolar manner. The budding pattern of the cell can be deduced from the positions of the bud scars, the protrusions visible on the surface of the cell. (B) shows a pseudohyphal cell with two bud scars visible at one pole, a conformation predicted by polar budding. (A) and (B) have the same scale, with the scale bar in (A) representing 1 μm.

C. $RAS2^{val19}$ Induction of Pseudohyphal Growth

A wild-type strain MATa/α ura3-52/ura3-52 (CGX31) was transformed with YCpR2V or YCp50 generating (A) CG69 and (B) CG71, respectively. These strains were streaked for single cells on SPHD medium, and resulting colonies were photographed after 33 hr of growth at 30°C. The scale bar represents 60 μm. Results are shown in Figure 10.

D. Invasiveness of S. Cerevisiae Pseudohyphae

A MATa/α Shr3 strain (F35) was streaked for single colonies on SPHD medium. After 2 days of growth at 300°C (A), a microcolony was photographed. Results are shown in

Figure 3. In (B), after 21 days of growth, pseudohyphae of a macrocolony were photographed. The pseudohyphae in (B) also represent a subclass of pseudohyphae that have unusually long cells. The scale bars in (A) and (B) represent 20 μm.

E. Production of Putative Blastophone-like Cells by S. Cerevisiae Pseudohyphal Cells

Strain F35 MATa/a HO/HO shr3-101/shr3-101 was streaked for single colonies on SPHD medium. Results are shown in Figure 11. After 3 days microcolonies like the one shown in (A) existed in the dense part of the streak-out. In these colonies the pseudohyphae were covered with small spherical yeast cells we putatively call blasto-conidia. These blastoconidia are on average 4 μm x 4 μm. Panel B shows an enlarged view of the pseudohypha at the right in (A).

EXAMPLE 3 Identification and Characterization of SHR3

A. Mutant Screen

Histidine is a non-catabolizable nitrogen source that is toxic at media concentrations greater than 1 mM. The mechanism underlying histidine inhibition is not known. Mutations affection vacuolar function may confer resistance to high concentrations of histidine since greater than 90% of the intracellular histidine is sequestered in this organelle. Spontaneous mutants resistant to histidine inhibition were isolated. Two precautions were taken in order to avoid the isolation of mutations which merely block the general uptake of amino acids and in particular histidine. First, the strain chosen for the isolation of histidine resistance was the non-reverting histidine

auxotroph PLY1(MATa his4Δ29 ura3-52), and must, therefore, obtain its histidine exogenously. Second, mutants were selected on SPD media (synthetic proline dextrose media + 30 mM L-histidine). On this media, in which proline is the sole nitrogen source, histidine enters the cell through several genetically distinct systems including the general amino acid permease (GAP1), the histidine specific permease (HIP1) and the arginine permease (CAN1). It is important to note that neither gap1 nor hip1 mutant strains grow on selective SPD+30 mM histidine media. Although on proline media no single permease mutation is capable of blocking histidine uptake, as shown herein, Shr3⁻ mutants define a common regulatory protein apparently affecting the function of all amino acid permeases.

Spontaneous Shr mutants were selected on SPD media supplemental with 30 mM histidine. SPD media contains four times the recommended amount of yeast nitrogen base (without amino acids and ammonium sulfate); this increased amount was necessary in order to minimize background growth. PLY1 cells pre-grown in YPD Media were harvested at a cell density of 2 X 10^7 cells ml^{-1}, washed twice and resuspended in sterile water. Cells were spread on selective media at cell densities between 10^5 and 10^7 cells per plate. Resistant colonies were picked and streaked for single colonies on SPD + 30 mM histidine. Twenty-three mutants giving rise to streaks of colonies of similar size and exhibiting uniform histidine resistance were selected for subsequent characterization. These strains were backcrossed to PLY4 (MATa his4Δ29 ura3-52 ade2Δ1::URA3). All of the isolated shr mutations were recessive; heterozygous diploid strains did not grow on SPD + 30 mM histidine. Tetrad analysis indicated that the mutant phenotypes

segregated 2:2. Complementation was examined in diploids resulting from all possible combinations of pairwise mutant by mutant crosses.

B. Genetic Characterization

Initial characterization of spontaneous super high histidine resistant (shr) mutants has shown that resistance results from recessive mutations in single genes defining nine complementation groups.

These complementation groups exhibit diverse phenotypes. Mutant representatives of five complementation groups, including shr3 mutants, grow poorly when grown under conditions known to effect vacuolar functions: these mutants are sensitive to hyperosmotic culture conditions and high Ca^{2+} concentrations. No temperature sensitive phenotypes were observed.

Attention was focused on the SHR3 complementation group because mutations in SHR3 exhibited the most striking phenotypes during the initial characterizations (please see the following sections for details). Mutant shr3$^-$ strains are inhibited by low but not by high concentrations of histidine. This bizarre sensitivity persists even in a HIS background. Mutant shr3$^-$ strains are sensitive to high media concentrations of Ca^{2+}. Mutations in SHR3 abolish histidine specific transport into vacuoles. Mutant shr3$^-$ strains have elevated levels of GCN4 even when grown under conditions that normally repress GCN4 expression. GCN4 is the transcriptional activator responsible for general control of amino acid biosynthesis in yeast. Increased levels of GCN4 indicate that cells are starved for at least one of the amino acids involved in the general control regulatory network.

C. Cloning of SHR3

The SHR3 gene was cloned by complementation of poor growth phenotype exhibited by shr3-23 cultured on SPD+1mM histidine. Strain PLAS23-4B (ura3-52, his4Δ29, shr3-23) was transformed with DNA from a plasmid yeast genomic library constructed in the YCp50 vector (Rose et al. Gene 60: 237-243 (1987)). Ura^+ transformants were selected and then replica plated onto SPD + 1mM histidine. Four out of 8000 Ura^+ transformants grew when transferred onto SPD+1mM histidine, and when subsequently tested these transformants were no longer resistant to 30 mM histidine, an expected result for complementation of a fully recessive mutation. Plasmids pPL152, pPL153, pPL154 and pPL155 were recovered from these strains. Each plasmid complemented all three shr3 alleles. Restriction endonuclease analysis of the plasmid insert DNA identified a common 8.4 kb fragment.

D. Plasmid constructions

Plasmids with inserts derived from pPL154 capable of complementing shr3 mutations were constructed as follows (Figure 1). Plasmid pPL164 was constructed by inserting the 11 kb BamHI fragment from pPL154 into BamHI digested pRS316 (Sikorski and Hieter, Genetics 122: 19-27 (1989)). Plasmid pPL164 was digested with EcoRI and religated; the resulting plasmid (pPL183) contains a 4 kb insert. Plasmid pPL179 was constructed by inserting the 3 kb EcoRI--KpnI fragment from pPL183 into EcoRI-KpnI digested pRS316. Plasmids pPL183 and pPL179 have the insert DNA cloned in opposite orientations. The 1.4 kb AccI fragment containing the SHR3 gene was isolated from pPL179 and the ends were filled in with Klenow fragment and inserted into

EcoRV digested pBSIISK(+) [Stratagene], creating plasmid pPL202. Plasmid pPL210 was constructed by inserting the 1.4 kb SalI-EcoRI from pPL202 into SalI-EcoRI digested pRS316.

A precise deletion allele of SHR3 was created by removal of the entire protein coding sequence and replacement of this segment with the selectable marker URA3. This construct, shr3Δ1::URA3, was created in two steps using the polymerase chain reaction (PCR). A 36 base synthetic single stranded DNA PCR primer (3-5′ΔH) that included 9 bases to create a HindIII site and 27 bases complementary to positions -24 through +3 with respect to the initiation ATG was synthesized. The 3-5′ΔH primer in conjunction with the T7 primer were used to prime a PCR reaction using plasmid pPL202 as template DNA. The amplified 450 bp fragment was digested with HindIII and XhoI and ligated into HindIII-XhoI digested pBSIISK(+) resulting in plasmid p5′Δ3. A second 53 base synthetic primer (3-3′ΔHX) was synthesized; it included 15 bases to create a HindIII site and an adjacent XhoI site, 38 bases homologous to the termination codon and the 35 bases 3′ to the coding region. The 3-3′ΔHX primer and the T3 primer were used to prime a second PCR reaction using pPL202 as template DNA. The amplified 350 bp fragment was digested with HindIII and EcoRI and ligated into HindIII-EcoRI digested p5′Δ3 creating pPL216 (shr3Δ3). Plasmid pPL219 (shr3Δ1::URA3) was constructed by inserting a 1.1 kb HindIII fragment containing the URA3 gene into the HindIII site of plasmid pPL216. Plasmid pPL130 was constructed by inserting a 6.2 kb BamHI fragment containing the ADE2 gene into BamHI digested pUC19 (Vieira and Messing, 1987). Plasmid pPL132 containing the ade2Δ1::URA3 deletion allele

was constructed by inserting the URA3 selectable marker into BglII digested pPL130.

Epitope tagging of SHR3 was performed as described by Kolodziej and Young (Methods Enzymol. 194: 508-519 (1991)) using site directed insertion mutagenesis (Kunkel et al. Methods Enzymol. 154: 367-382 (1987)). A nine amino acid epitope from the influenza virus hemagglutinin protein HA1 (Wilson et al. Cell 36: 767-778 (1984)) was introduced into the SHR3 sequence between amino acid residues 171 and 172 (SHR3::FLU2). A synthetic oligomers with 27 nucleotides encoding the HA1 epitope flanked on each side by 20 bases of complementary SHR3 sequence was synthesized. This oligomer was annealed to single stranded pPL210 DNA prepared with helper phage M13K07 (Vieira and Messing Methods Enzymol. 153: 3-11 (1987)) in the dut^- ung^- E. coli host, RZ1032 (Kunkel et al. Methods Enzymol. 154: 367-382 (1987)). After elongation, ligation, and transformation into dut^+ ung^+ host, plasmid DNAs were screened for the presence of a new AatII restriction site diagnostic for successful mutagenesis. Plasmid pPL230, containing the epitope tagged SHR3::FLU2 construct, complements all shr3 mutations.

Plasmid pPL247 was constructed by inserting the 3.5 kb SalI-SpeI fragment containing the GAP1 gene (isolated from pMS16) into SalI-SpeI digested pRS316. The nine amino acid HA 1 epitope was independently introduced into two locations within the GAP1 sequence, between amino acid residues 62 and 63 (GAP1::FLU1) and amino acid residues 550 and 551 (GAP1::FLU2). The resulting plasmids pPL257 and pPL258 containing these epitope tagged constructs complemented the growth defects of a gap1 null mutant strain. Plasmids pPL262, pPL269 and pPL28s were construct-

ed by inserting the 3.5 kb SalI-XbaI inserts from pPL247, pPL257 and pPL258 into SalI-XbaI digested YCp405, respectively (Ma et al., Gene 56: 201-216 (1987)).

E. DNA and Predicted Protein Sequences of the SHR3 Gene

The nucleotide sequence of the SHR3 gene was determined by DNA sequence analysis of the 2.7 kb genomic EcoRI-KpnI fragment (Figure 4). Nested deletions of the insert fragments of plasmids pPL183 and pPL179 were generated by digestion with ExoIII as described by Henikoff (Gene 28: 351-359 (1984)) except that ExoVII was substituted for S1 nuclease. Double stranded DNA was prepared as described by Haltiner et al. (Nucleic Acids Res. 13: 1015-1026 (1985)) and sequenced by the dideoxy chain termination method (Sanger et al. Proc. Natl. Acad. Sci. 75: 5463-5467 (1977)). The nucleotide sequence of the 1.4 kb AccI fragment capable of complementing shr3⁻ mutations is shown in Figure 5.

The SHR3 open-reading frame beginning with the initiation codon ATG is comprised of 626 bp. The location of the open reading frame corresponds to that predicted by endonuclease mapping analysis. The SHR3 open reading frame encodes a protein comprised of 209 amino acids with a molecular mass of 23.5 kDa and a pI = 10.04 (Figure 5) (Finer-Moore et al., in Prediction of Protein Structure and the Principles of Protein Conformation (G. Fasman, ed., Plenum Press, New York)). The SHR3 protein is predicted to be an integral membrane protein comprised of four membrane spanning domains and an extremely hydrophilic carboxy terminal domain (see Figure 6 for hydropathy plot). Twenty-four of the last 48 amino acids in

the carboxy terminal domain are charged: they include 8 acidic residues and 16 basic residues. The carboxy-terminal domain is predicted to be exposed to the cytoplasm (Hartmann et al., PNAS USA 86 5786-5790 (1989)) and to adopt an α-helical secondary struture (Finer Moore et al.). SHR3 showed no significant homology with any proteins in the PIR, SwissProt, and GenPept (translated GenBank) protein data bases. Protein homology searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service (Altschul et al., J. Mol. Biol. 215: 403-410 (1990)).

F. Mapping of SHR3

SHR3 was mapped by hybridization to whole yeast chromosomes separated by pulse field electrophoresis (Carle and Olson Proc. Natl. Acad. Sci. 82: 3756-3760 (1985)). Full length chromosomes isolated from yeast strains with fragmented chromosomes VII (Vollrath et al. Proc. Natl. Acad. Sci. 85: 6027-6031 (1988)) and chromosomes digested with NotI and SfiI (Link and Olson Genetics 127: 681-698 (1991)) were electrophoretically separated, transferred to a nitrocellulose and hybridized to a radioactively labeled probe specific to SHR3. The probe hybridized to sequences located on the extreme left arm of chromosome IV (~130kbp NotI fragment). Data from these chromoblots and additional Southern blot experiments at both low and high stringency indicate that the SHR3 gene is present as a single copy in the haploid yeast genome.

To determine the precise chromosomal location of SHR3, three point crosses involving known markers on the left arm of chromosome IV were carried out. Subsequent tetrad analysis established the gene order and map dis-

tances (centimorgans) as follows: CEN IV-cdc9(13cM--arf1-(10 cM-shr3-(>50 cM)-cdc13.

EXAMPLE 4 Biochemical Characterization of shr3⁻ Mutants

A. Disruption of SHR3

Diploid yeast strain AA305 (HIS3/his3Δ200,LEU2/leu2-3,112,ura3-52/ura3-52,lys2/lys2,ade2/ade2) was transformed with the construct shr3Δ1::URA3. Stable Ura$^+$ transformants were selected and sporulated. Tetrads were dissected on both YPD and SD media (minimal media supplemented only with auxotrophic requirements). Spore viability was excellent on SD media. When transferred to YPD, spore-derived colonies containing the shr3 deletion and auxotrophies for either histidine or leucine did not grow. The synthetic lethality of shr3 null mutations in combination with these auxotrophic alleles was reflected in the pattern of spore inviability observed on YPD. These results show that on YPD amino acid auxotrophic strains require SHR3 function during both spore germination and vegetative growth. Similar synthetic lethality was observed when auxotrophic shr3 null mutant strains were transferred to SC, a medium with high concentrations of all amino acids. A summary of our results regarding combinations of amino acid auxotrophic alleles and synthetic lethality with shr3 null mutations is presented in Table 4. These genetic data indicate that SHR3 is required for the uptake of other amino acids in addition to histidine. Genetic analysis revealed that null shr3 mutations are lethal in either his⁻ or leu⁻ backgrounds.

Table 4. Synthetic Lethallty on Complex Media[a]

Double Mutant		Viability
shr3a 1::URA3	leu2	-
	his3	-
	trp 1	-
	ilv1	+
	lys2	+
	arg4	+
	ade2	+

[a]Germination and Growth on YPD and SC Media

Subsequent dissections on minimal media were carried out and four viable spores from each tetrad were observed. Thus, the synthetic lethality previously observed was due to the rich nutrient environment of YPD media. When these spore derived colonies from minimal media were replica plated onto SPD + 39 mM histidine media, a 2:2 segregation pattern was observed. Histidine resistance was 100% linked to the URA3 deletion marker, indicating that the SHR3 leads to the resistance phenotype. Disruption of the SR3 locus was confirmed by Southern blot analysis of DNA obtained from histidine resistant colonies. Southern blot experiments at both low and high stringency indicate that the SHR3 gene is present as a single copy in the haploid yeast genome. When the spore derived colonies from minimal media were replica plated onto YPD media, the synthetic lethality phenotype was again observed.

The viability of auxotrophic shr3 null mutant strains on SD medium must reflect the uptake of required amino acids through residual permeases present in greatly reduced amounts, or by non-specific uptake systems. We surmise that the observed synthetic lethality on both YPD and SC media is due to their high amino acid content. On these media the overabundance of competing amino acids must interfere with the residual uptake mechanisms, effectively inhibiting uptake of the required amino acid. Thus, when grown on either YPD or SC, auxotrophic shr3 null mutants cannot synthesize required amino acids nor can they import them from the external environment. Similar YPD synthetic lethality has previously been observed for mutations that pleiotropically affect amino acid uptake (Garrett, J. Gen. Microbiol. 135: 2429-2437 (1989); McCusker and Haber, Mol. Cell. Biol. 10: 2941-2949 (1990)). Synthetic lethality was not observed with the original shr3 mutant alleles isolated (shr3-3, 3-16, and 3-23), suggesting that these mutations are not complete loss of function alleles.

B. Shr3 is Allelic to apf (also known as aap)

Previously isolated mutations known as apf and aap pleitropically effect amino acid transport in yeast (Surdin et al., Biochim. Biophys. Acta 107: 546-566 (1965) and Grenson and Hennaut, J. Bacteriol. 105: 477-482 (1970)). It has been shown that apf and aap mutations are allelic (Grenson and Hennaut). Mutant apf strains, initially isolated as being DLparafluorophenylalanine resistant, were subsequently found to be resistant to a variety of other toxic amino acid analogues. This resistance was shown to result from a reduction in transport activity of

multiple amino acid permeases including the general amino acid permease (GAP1) and many specific high affinity amino acid permeases. These findings suggest that there is a common pathway or maturation process that is required for the functional expression of amino acid permeases in yeast.

Apf mutant strains grew well on SPD media supplemented with 30mM histidine. This was surprising since it had been reported that apf mutant strains are unable to grow on medium containing proline as the sole nitrogen source (Grenson and Hennaut). A complementation test of the apf mutation and a shr3 mutation was carried out. Strain CGAS53-2E (MATa, apf, ura3-52) was mated to strains PLAS16-4B (MATα, shr3-16, ade2Δ1::URA3, ura3-52, his4Δ29) and PLAS16-6C (MATα, SHR3, ade2Δ1::URA3, ura3-52, his4Δ-29). PLAS16-4B and PLAS16-6C are isogenic except at the SHR3 locus. Diploids derived from CGAS53-2E x PLAS16-4B were resistant to 30 mM histidine and grew poorly on 1 mM histidine indicating that these two mutations do not complement. Transformants of strain CGAS53-2E transformed with a plasmid containing the SHR3 gene (pPL210) were unable to grow on SPD + 30 mM histidine but grew well on SPD + 1mM Histidine. These results indicate that apf and shr3 mutations are allelic.

Based on our genetic analysis (Table 4) and amino acid uptake studies (Table 5 below) with shr3 null mutant strains, as described herein, and previous studies with apf strains (Grenson and Hennaut), at least 11 genetically distinct amino acid permeases require SHR3 for functional expression. The permeases affected by mutations in SHR3 are the general amino acid permease (GAP1), histidine (HIP1), proline (PUT4), arginine (CAN1), glutamate (dicar-

boxylic acid amino permease), lysine, leucine, methionine, serine, valine, and tryptophan.

C. Homozygous Shr3⁻ Diploid Strains Form Pseudohyphae

During complemenation testing with apf mutant strains it was noted that upon prolonged incubation on SPD + 1 mM histidine homozygous shr3⁻ diploid strains developed a distinctive hairy colony morphology. This morphology, which results from the extensive formation of pseudohyphae, was shown to be dependent upon inactivation of the SHR3 gene.

D. Amino acid uptake and transport

Using purified vacuolar membrane vesicles, Sato et al. (J. Biol. Chem. 259: 115 (1984)) have demonstrated that amino acids are transported into the yeast vacuole through seven independent proton/ amino acid antiport systems. Arginine is transported via two different antiport systems (K_t = 0.4 and 1.5 mM, respectively); histidine is transported via a single antiport system (K_t = 1.2 mM). Ohsumi et al. (J. Bacteriol. 170: 2676-2682 (1988)) have shown that appropriate concentrations of Cu^{2+} ion breaks down the permeability barrier of plasma membranes but not the vacuolar membranes of yeast cells. The selective effect of Cu^{2+} on the plasma membrane makes it possible to assay vacuolar function in situ. Vacuolar transport rates observed with Cu^{2+} treated cells are similar to isolated vacuole membrane vesicles. Amino acid uptake was assayed essentially as described by Ohsumi et al. (1988). Exponentially grown cells were harvested, washed twice with water and resuspended to a density of 2 x 10^8 cells

ml^{-1} in AAB buffer (10 mM MES-Tris pH 6.4, 2 mM $MgCl_2$, 0.6 M sorbitol supplemented with 10 mg ml^{-1} cycloheximide). The cell suspension was equilibrated to 30°C and uptake was initiated by the addition of radiolabeled amino acids. Subsamples (100 μl) were withdrawn, diluted into 3 ml of ice cold MB buffer, filtered through Whatman GF/F filters, and washed 3X with 5 ml ice cold MB buffer. Filter discs were allowed to dry and radioactivity was measured by liquid scintillation counting.

For kinetic analysis of histidine, proline, arginine and citrulline three different [^{14}C]-amino acid stock solutions (0.25 mCi $mmol^{-1}$, 1.25 mCi $mmol^{-1}$, or 125 mCi $mmol^{-1}$ (for citrulline a 55.9 mCi $mmol^{-1}$)) were used to obtain amino acid concentrations ranging from 10 to 0.002 mM. The uptake rate of lysine, glutamate, leucine and adenine were determined at 10 mM and 0.004 mM substrate concentrations; two [^{14}C]-labeled substrate stock solutions (0.25 mCi $mmol^{-1}$ and 125 mCi $mmol^{-1}$) were required to obtain desired final concentrations. The initial uptake rates were determined at each substrate concentration; subsamples were removed at 30, 90 and 180 sec, filtered and washed as described. The uptake rate for every amino acid was linear throughout the subsampling period. Cell protein was determined by the method of Markwell et al. Anal. Biochem. 87: 206-210 (1978) in samples of cells boiled in 0.1 M NaOH. Uniformly ^{14}C-labeled L-amino acids and adenine were obtained from Amersham Corporation, Arlington Heights, IL; L-[Ureido-^{14}C]-citrulline was obtained from NEN, DuPont Company, Wilmington, DE.

Histidine and arginine uptake into wild-type and mutant shr strains was assayed using saturating concentrations of amino acids, i.e. 12 mM histidine, and 4 mM

arginine, respectively. All three members of the shr3 complementation group (shr3-3, shr3-16 and shr3-23) exhibited less than 10% of wild-type histidine transport, whereas arginine uptake appeared relatively unaffected. Cu^{2+} treatment always resulted in stimulated arginine uptake. None of the representatives of the other complementation groups exhibited a defect in histidine uptake as severe as that shown by shr3 mutants. Apparently, the decreased rates of histidine uptake into these shr3 mutants is sufficient to support the growth of His^- auxotrophs, but insufficient to permit internal histidine concentrations to reach toxic levels. Histidine uptake into whole cells matched the rate of vacuolar transport after Cu^{2+} treatment in both mutant and wild type cells. Studies on null alleles of shr3 suggested that the viability of $Shr3^-$ His^- double mutants is characteristic only of leaky mutant alleles of SHR3 (see subsequent section). Strains carrying shr3 mutations in either a His^+ or His^- background fail to grow well on SPD + 1 mM histidine but grow well on SPD + 30 mM histidine.

Vacuolar pH in the wildtype and mutant $shr3^-$ strains was determined and shown to be the same in all strains. Therefore, the alterations in the observed rates of amino acid transport did not result from changes in the energization state across the vacuolar membrane. Consistent with our observations, there were no gross changes in vacuolar morphology.

For further analysis of amino acid transport, the kinetics of histidine uptake into isogenic wild-type and shr3Δ1::URA3 mutant strains were examined. Strains were pregrown in media containing urea as the sole nitrogen source; both wild-type (PLY143) and shr3Δ1::URA3 mutant

(PLY152) strains grow at similar rates in this media (2.5 hr doubling time). Urea is a non-repressing nitrogen source, and cells express maximal levels of amino acid permeases. Figure 2 shows a double reciprocal plot of the histidine uptake data. The plot is clearly non-linear, indicating, as expected from previous work, that histidine uptake is mediated by multiple permeases with different affinities. At every concentration examined the initial rate of histidine uptake was significantly reduced in the shr3 deletion mutant as compared to the wild-type strain. These data indicate that in the complete absence of SHR3, the amino acid transport activity of multiple permeases is reduced. Similar kinetic data were obtained for proline, citrulline and arginine.

The analysis of the high and low-affinity components of uptake indicates that the deletion of SHR3 did not result in large changes in the apparent affinity constant for transport (K_t) of either the high or low-affinity transport systems (high affinity, SHR3 K_t = 10 μM vs shr3-Δ1 K_t = 6 μM; low affinity, SHR3 K_t = 4 mM vs shr3Δ1 K_t = 8 mM). These data suggest that the decreased rates of amino acid uptake in shr3 null mutants is not due to altered affinities of the permeases, but rather results from a reduction in the concentration of functional permeases.

Table 5 provides a summary of the amino acid transport and adenine uptake rates determined for wild-type and shr3 null mutant strains grown in SUD. The uptake rates were determined at two substrate concentrations. At high substrate concentrations (10 mM), amino acid uptake occurs predominantly through GAP1; at low substrate concentrations (0.004 mM) uptake occurs via the specific amino acid permeases. The data clearly show the pleiotropic

effect of the shr3 null mutation on both general and specific amino acid uptake systems. The uptake of each of the amino acids we examined was reduced in shr3 null mutant strains. Adenine uptake was relatively unaffected, indicating that the expression of the purine-cytosine permease (FCY2), which is not a member of the yeast amino acid permease gene family (Weber et al., J. Mol. Evol. 27 (34): 1-350 (1988); Weber et al., Mol. Microbiol. 4: 585-596 (1990)), is not dependent upon SHR3 function.

Table 5. Amino Acid and Adenine Uptake into SHR3 and shr3 Null Mutant Strains

	High Substrate Concentration (10 mM)[a]			Low Substrate Concentration (0.004 mM)[b]		
Substrate	SHR3	shr3Δ1	Fold Decrease	SHR3	shr3Δ1	Fold Decrease
Citrulline	0.113	0.064	1.8	0.572	0.0156	37.0
Glutamate	4.490	0.618	7.3	25.0	1.20	20.8
Leucine	3.795	0.834	4.6	15.0	0.790	19.4
Proline	0.124	0.023	5.4	0.135	0.0198	6.8
Histidine	1.504	0.397	3.8	51.0	10.0	5.1
Lysine	0.279	0.191	1.5	9.05	3.62	2.5
Arginine	0.494	0.157	3.2	102.0	45.0	2.3
Adenine	0.122	0.116	1.1	12.0	8.94	1.3

[a]rate = nmol min^{-1} mg^{-1} protein
[b]rate = pmol min^{-1} mg^{-1} protein

E. Cellular Consequences of a General Block in Amino Acid Transport

To examine the effect of shr3 mutations on general amino acid control we determined the level of GCN4 expression in isogenic histidine auxotrophic Shr^+ (PLAS1-7B) and

Shr⁻ (PLAS23-4B) strains. Amino acid starvation induces the expression of GCN4, the general transcriptional activator of genes in several amino acid biosynthetic pathways (Hinnebusch, Microbial. Rev. 52:248-273 (1988)). Strains were transformed with a reporter plasmid construct (p180) containing GCN4-LacZ with the natural leader sequence that places GCN4 under general control in the presence of AUG codons (Hinnebusch, Mol. Cell Biol. 5: 2344-2360 (1985)). In parallel control experiments, strains were transformed with a constitutively expressed gcn4-LacZ construction (p227) (Mueller and Hinnebusch, Cell 46: 201-207 (1986)). Overnight cultures of these histidine auxotrophic strains transformed with β-galactosidase (LacZ) vectors p180 and p227 were grown in complete synthetic media lacking uracil (SC - ura). Cells were diluted 1:5 with either SC - ura for repressing conditions or SC lacking both uracil and histidine (SC - ura,his) for derepressing histidine starvation conditions. Freshly diluted cultures were allowed to grow for an additional 5 hr at 30°C and LacZ activity was determined as described by Rose et al. (Proc. Natl. Acad. Sci. USA 78:2460-2464 (1981)). Enzymatic activities were normalized to soluble protein concentrations determined for each extract by the method of Bradford (Anal. Biochem. 72:248-254 (1976)).

Under repressing conditions, in the presence of all amino acids, mutant shr3 strains express two-fold more β-galactosidase activity than wild-type cells (Figure 14A, Repressing). These results indicate that shr3 mutant cells sense starvation conditions even when grown in the presence of excess amino acids. Under conditions of histidine starvation (derepressing), shr3 strains express GCN4-LacZ at very high levels (Figure 13A, DR-his). The high levels of

GCN4 expression are comparable to those found for gcd1 mutations (Hinnebusch, Mol. Cell Biol. 5: 2344-2360 (1985)), an observation demonstrating that shr3 mutants are hypersensitive to amino acid starvation. In the control experiments both wild-type and mutant strains expressed similar levels of GCN4-lacZ activity (Figure 13B). These control experiments indicate that shr3 mutations do not enhance the stability of GCN4-LacZ.

Mutant shr3 cells exhibit greatly reduced growth rates in media containing proline as the sole nitrogen source: exponentially growing Shr+ cells double every 10 hours, whereas the doubling time of shr3 null mutants is increased to over 25 hours. The slower growth of shr3 mutants on proline medium must reflect nitrogen source limitation since mutant cells transport proline at greatly diminished rates (see Table 5). Diploid strains of S. cerevisiae undergo dimorphic transitions (Gimeno et al., Cell 68:1077-1090 (1992); Gimeno and Fink, Science 257:626 (1992)). Compared to isogenic wild-type diploids, homozygous shr3 diploids growing on proline medium undergo dimorphic transitions at enhanced frequencies. Since shr3 mutations impair proline transport and induce starvation responses (Figure 13), the observation that these mutations enhance pseudohyphal growth strongly suggested that nitrogen source availability regulates the dimorphic transition. This model was proven by the observation that wild-type diploids could be stimulated to undergo dimorphic transitions when grown in media containing limiting concentrations of ammonia as the sole nitrogen source (Gimeno et al., Cell 68:1077-1090 (1992)). The enhanced pseudohyphal growth, like the elevated GCN4 levels, is an indication of the in vivo consequences of reduced amino acid uptake.

F. SHR3 is a Component of the Endoplasmic Reticulum (ER)

A functional epitope tagged SHR3 allele was constructed as previously described (see section on plasmid construction). The intracellular location of SHR3 was determined by immunolocalization of a functional epitope tagged SHR3 protein by fluorescence microscopy. Strain PLAS23-4B (shr3-23, ura3-52, his4Δ29) was transformed with a centromere-based plasmid containing the epitope tagged SHR3 construct (pPL230). The Ura+ transformants containing this plasmid were no longer histidine resistant and grew well on SPD + 1 mM histidine, indicating that the epitope tagged SHR3 protein fully complements the shr3- mutation. Cells were pre-grown to a density of 1 x 10^7 cells ml^{-1} in complete synthetic media minus uracil (CSD - ura) in order to select for plasmid maintenance. These pre-grown cells were then diluted to a density of 2 x 10^6 cells ml^{-1} in YPD media and grown for 5 hr. Cells were fixed, spheroplasted and incubated with antibodies essentially as described by Davis and Fink (Cell 61: 965-978 (1990)). Control cells transformed with pPL210 (untagged SHR3) were prepared in parallel.

Cells transformed with the epitope tagged SHR3 construct, but not with the control plasmid, showed bright perinuclear rim-staining that often extended in a filamentous manner into the cytoplasm. The SHR3 immunofluoresceance staining pattern is the same in both SHR3 and shr3 null mutant strains. The perinuclear staining pattern observed is essentially identical to that observed for the lumenal ER protein KAR2 (Rose et al., Cell 57: 1211-1221 (1989)) and ER membrane protein SEC 62 (Deshiaies and Sheckman, J. Cell Biol. 105: 633-645 (1987)). Based on electron microscopic analysis the yeast ER and nuclear envelope and

are thought to be continuous (Novick et al., Cell 21: 205--215 (1980)). Therefore the immunofluorescence staining pattern observed with epitope tagged SHR3 is consistent with localization of the protein to the ER membrane. It should be noted that within the hydrophilic carboxyterminal domain of SHR3 there are two -K-K-X-X- sequences and two lysine residues at the extreme carboxy-terminus. Similar sequence motifs have been shown to be sufficient for ER-retention in mammalian cells (Nilsson et al., Cell 58:707-718 (1989)).

G. Null shr3⁻ Mutants Accumulate GAP1 in the ER

The intracellular location of SHR3 in the ER suggested a possible role for SHR3 in the processing of amino acid permeases within the ER. To test this hypothesis, functional epitope tagged versions of the general amino acid permease (GAP1::FLU1 and GAP1::FLU2) were constructed. These constructs were used to compare the intracellular location of GAP1 in wildtype and shr3⁻ deletion strains. In wildtype cells GAP1 showed a plama membrane rim-staining pattern, the result expected for a plasma membrane protein. In the shr3 deletion strain, GAP1 staining was perinuclear, a pattern identical of that of SHR3. These results indicate that in the absence of SHR3, GAPΔ fails to localize to the plasma membrane and has an intracellular distribution consistant with localization in the ER. It should be noted that since GAP1 gets into the ER in the complete absence of SHR3, SHR3 is not required for entry into ER. Thus, it appears that SHR3 is required for the efficient transit of GAP1 through the secretory pathway to the plasma membrane. It is also possible to determine whether or not shr3⁻ mutations affect the intracellular localization of the histidine specific permease (HIP1).

Analysis of membrane preparations from SHR3 and shr3 strains provides important insights into the nature of the defect in shr3 strains. Membranes were isolated from SHP3 and shr3 strains (see Example 1, Experimental Procedures) containing GAP1 tagged with an epitope near its amino terminus (GAP1::FLU1). The levels of GAP1 in total cell extracts and in the isolated membranes were the same in SHR3 and shr3 strains (less than 15% variation) as estimated by quantitative immunoblots. The membrane preparations from SHR3 and shr3 strains were treated with a variety of reagents to ascertain the nature of the association between GAP1 and the membranes. The results showed that GAP1 is not extracted by reagents known to extract peripherally associated membrane proteins, but is extracted in both SHR3 and shr3 by a nonionic detergent known to solubilize integral membrane proteins (data not shown). These results indicated that roughly equivalent amounts of GAP1 is localized to membranes in SHR3 and shr3 and suggest that SHR3 does not alter the insertion of GAP1 into those membranes. The topology of GAP1 within the membrane preparations obtained from wildtype and shr3 deletion strains was examined by limited protease digestion. In the membranes obtained from shr3, GAP1 is more susceptible to trypsin digestion and, at dilute trypsin concentrations, gives a digestion pattern different from GAP1 in SHR3 strains. In SHR3 membranes fragment a predominates, whereas in mutant membranes trypsin digestion products b and d predominate (data not shown). Similar results were obtained with membrane preparations obtained from strains expressing GAP1::FLU2, a GAP1 protein tagged near the carboxy terminus. The increased protease sensitivity of GAP1 in shr3 deletion strains suggests that in the absence of SHR3 permeases have an altered topology. The observed variation in protease

sensitivity could be due to altered folding or to different local environments, i.e., in wild-type strains GAP1 is primarily associated with the plasma membrane, whereas in shr3 deletion strains GAP1 is in the ER membrane.

H. The ER Block In shr3 Mutants Is Specific For Amino Acid Permeases

To ascertain whether the observed ER export block was general or restricted to amino acid permeases, we examined the intracellular distribution and processing state of several proteins that require passage through the secretory pathway. We compared the intracellular location of the plasma membrane H^+-ATPase (PMA1) in wild-type and shr3 null mutant strains by immunofluorescence microscopy. PMA1 is an integral polytopic membrane protein component of the plasma membrane comprised of at least eight transmembrane domains (Serrano et al., Nature 319:689-693 (1986)). The immunolocalization of PMA1 in SHR3 and shr3 strains was indistinguishable; in both strains a faint stain highlighting the external surface of the cells was observed (data not shown). SHR3 is apparently not required for the processing and correct intracellular targeting of PMA1 to the plasma membrane.

The intracellular processing state and secreted amounts of α-factor in MATα wild-type and shr3 null mutant cells are the same (data not shown). These results are consistent with bioassays analyzing halo sizes on tester lawns of MATa cells. Additionally, MATa shr3 null mutant cells secrete similar levels of a-factor and are equally sensitive to α-factor as isogenic MATa SHR3 cells. These results indicate that shr3 mutations do not have a general effect on secretion or membrane internalization. These conclusions are

supported by studies examining the processing of invertase in shr3 mutants (data not shown). In wild-type cells invertase becomes extensively glycosylated as it passes through the various Golgi compartments. As a consequence of extensive outer chain glycosylation, invertase runs as a heterogeneous high molecular weight smear upon electrophoresis. After treatment with endoglycosidase H (Endo H) the resulting unglycosylated form runs as a single band (Franzusoff and Schekman, EMBO J. 8:2695-2702 (1989)). The results indicated that invertase processing is the same in wild-type and shr3 null mutant cells and that the addition of outer chain glycosylation occurs in an SHR3 independent manner.

The processing and intracellular targeting of the vacuolar protease carboxypeptidase Y (CPY) in wild-type and shr3 null mutant cells is also identical. In wild-type cells, preproCPY enters the secretory pathway by translocation across the ER membrane. In the ER the signal sequence is cleaved and proCPY becomes core glycosylated resulting in the 67 kDa P1 form. Outer chain glycosylation occurs within the Golgi, generating a 69 kDa P2 form. Finally, the mature 61 kDa CPY is formed after proteolytic processing in the vacuole (Stevens et al., Cell 30:439-448 (1982)). The results showed that there is no detectable difference between the intracellular processing or vacuolar targeting of CPY in wild-type and shr3 null mutant cells. Additionally, CPY was exclusively targeted to the vacuole; no extracellular CPY was detected in either wild-type or shr3 null mutant culture supernatants (data not shown). These results are consistent with our observations that mutations in SHR3 do not affect the vacuolar pH or vacuolar morphology (Preston et al., Proc. Natl. Acad. Sci. USA 86:7027-7031 (1989)).

I. SHR3 mutations do not generally affect the secretory pathway

Several experiments indicate that null shr3⁻ mutants correctly localize the plasma membrane H^+-ATPase (PMA1) and normally process α-factor, carboxypeptidase Y and invertase. First, the distribution of the plasma membrane H^+-ATPase in wildtype and null shr3⁻ mutant cells is similar (Figure 10). Also, wildtype and null shr3⁻ mutant cells process and secrete similar amounts of α-factor (Figure 11). Furthermore, null shr3⁻ mutant cells process and target carboxypeptidase Y and invertase correctly (Figure 12).

EXAMPLE 5 Method of Isolating Dimorphism Genes from Pathogenic Fungi

This method requires two materials: a dimorphic MATa/α diploid strain of Saccharomyces cerevisiae with an auxotrophic marker, and low ammonia (SLAHD) solid medium in petri dishes.

First, a plasmid library is generated from the fungus of interest. This library may be of one of two types. The first type of library is one which contains genomic DNA from the fungus of interest inserted in a S. cerevisiae vector with the 211 origin of replication which confers high copy number on the plasmid. This type of library may be used when S. cerevisiae is known to be able to use promoters from the particular fungus (for instance, many Candida albicans genes can be expressed in S. cerevisiae from their own promoters). The second type of library can always be used and is a complementary-DNA (cDNA) library made from the fungus of interest in a S. cerevisiae vector in which cDNAs are cloned next to a galactose inducible promoter. They are overexpressed by growing the yeast on galactose medium.

Using standard techniques, the dimorphic S. cerevisiae strain is transformed with the library and transformants are selected on selective plates. These transformants are screened three different ways. After 4-7 days of growth water is added to the transformation plate's and the transformants are resuspended in it. The resuspended transformants are plated on SLAHD plates (containing galactose and raffinose if the library is a galactose regulated cDNA library) at a density of 2000 colony forming units (cfus) per plate and 200 cfus per plate. The number of transformants that must be screened must be determined for each library and is usually 20,000 -100,000.

After 3-4 days the plates plated at a density of 2,000 cfus per plate are visually screened under a dissecting microscope and colonies with enhanced pseudohyphal growth are identified by their fuzzy morphology. Normal colonies are not fuzzy under these conditions but are symmetrical and round. These colonies are picked and the library plasmids the cells in them contain are isolated by standard techniques. These plasmids are reintroduced into the original S. cerevisiae strain used for the screen to ensure that they confer the enhanced phenotype. Once this has been shown either a positive activator of dimorphism or a gene with an important but indirect role in dimorphism has been isolated.

After 7 days the plates with 200 cfus plated on them are scored under a dissecting microscope. Under these conditions normal colonies are fuzzy. Colonies with suppressed pseudohyphal growth can be identified because they are not fuzzy but are symmetrical and round. As already explained library plasmids are isolated and tested to see if the repressed dimorphism phenotype is associated with the plasmid. Once this has been shown either a repressor of

dimorphism or a gene with an important but indirect role in dimorphism has been isolated.

In a third screen the transformation plates from above are rinsed with sterile water (after their transformants have been resuspended for the two screens above) and the plates are screened under the dissecting microscope for colonies that remain imbedded in the agar. Normal colonies do not remain imbedded in the agar. These colonies are screened under higher magnification to determine the morphology of their constituent cells. Colonies with agar imbedded filaments of cells, indicating that dimorphism was activated in the colony, are picked. Their plasmids are analyzed as above and shown to confer the enhanced dimorphism phenotype on the original strain that was transformed for the screen. The fungal gene on the library plasmid is either a positive activator of dimorphism or a gene with an important but indirect role in dimorphism. This strategy works for a genomic library in a 2μ vector. If a galactose-promoted cDNA library is used, transformants are selected on SC medium lacking uracil, resuspended in water, and then plated at a density of 300 cfus per plate on SC plates lacking uracil and with galactose and raffinose as sole carbon sources (to overexpress the cDNAs). After colony growth, screening proceeds as above.

Once a gene is isolated in this manner it can be analyzed at the molecular level and it can be studied in its organism of origin.

EXAMPLE 6 Isolation of a Pseudohyphal Gene in S. cerevisiae

A. Genetic Screen to Identify Positive Regulators of Pseudohyphal Growth

Because PHG is a MATa/α diploid specific phenomenon, a gene overexpression approach (reviewed in Rine, 1991) was chosen to identify PHG regulatory genes instead of a more classical mutagenesis strategy.

The wild-type Σ1278b strain CGX69 (MATa/α ura3-52/ura3-52) was transformed with a genomic *Saccharomyces cerevisiae* library (Connelly and Hieter, unpublished data) constructed in a URA3 marked 2μ based high copy vector. 15,000 transformants were obtained in 20 independent pools by selection on SC plates lacking uracil. After 5 days, transformants were resuspended in water, and plated at a density of 2,000 colony forming units per plate on SLAHD medium. During the next 3 days of the SLAHD plates were screened under a dissecting microscope. Most microcolonies were symmetrical and smooth, but at a frequency of about 4.5/1000, colonies with rough outlines composed of pseudohyphal filaments were observed (Figures 1A and 1B). Cells from these rough colonies were collected and nine different library plasmids that conferred enhanced PHG on CGX69 grown on SLAHD were isolated from them. A plasmid termed pCG7, isolated independently three times, conferred the most dramatically enhanced PHG on CGX69 grown on SLAHD and was chosen for further characterization. The gene on this plasmid responsible for the enhanced PHG phenotype was named PHD1 (pseudohyphal determinant).

B. Use of a Galactose Inducible cDNA Library to Clone Dimorphism Gene

The screening method described above is useful for isolating dimorphism genes from pathogenic fungi if their genes can be expressed in *S. cerevisiae*. cDNA libraries can be made from any fungus in a *S. cerevisiae* expression vector. This method is modified to allow a cDNA library to be screened with it. CGX69 was transformed with a galactose inducible *S. cerevisiae* cDNA library (Lieu *et al.*, in press) and transformants were selected on solid SC medium lacking uracil. Exactly as above, the transformants were pooled and plated on SLAHGR (identical to SLAHD plates except that they contain 2% galactose and 1% raffinose are sole carbon sources) and scored. 29,000 transformants were screened in this manner. After plasmid rescue and retransformation experiments 9 plasmids were obtained that conferred highly enhanced pseudohyphal growth on CGX69 growing on SLAHGR medium but not on SLAHD medium (the glucose in SLAHD medium represses expression of the cDNA). The 5′ ends of these 9 cDNAs were sequenced.

One of these cDNAs encoded PRO1, the first enzyme in the proline biosynthetic pathway (Li, W., and Brandriss, M.C., *J. Bacteriol*, *174*:4148-4156, (1992)). Two of these cDNAs encoded SSB1, a nucleolar single-stranded nucleic acid binding protein (Jong, *et al.*, *Mol. Cell. Biol.*,7:2947-2955,(1987)). Three of these cDNAs encoded BMH1, a putative negative regulator of protein kinase C (van Heusden, *et al.*, *FEBS Lett.*,*302*:145-150, (1992)). One of these cDNAs appears to encode the yeast homolog of ribosomal protein S12 (Lin, *et al.*, *J. Biol. Chem.*, *262*:14343-14351, (1987)). The sequence of the other two cDNAs was not informative. This approach has identified *S. cerevisiae* genes with probably both direct and indirect roles in dimorphism. The modified

screening method has been validated and can be used to identify dimorphism genes from pathogenic fungi.

C. Plasmid Construction

Plasmids used in this study are listed in Table 3. pCG7 is the plasmid identified in our screen containing PHD1 isolated from a high copy genomic library. This genomic library was made in pRS202 (Connelly and Hieter, unpublished data), a derivative of pRS306 (Sikorski, R.S., and Hieter, P., *Genetics*, *122*:19-27, (1989)) that contains the 2 μ origin of replication in the AatII site and BgIII linkers in the SmaI site of the polylinker. The library was made by cloning size selected fragments (6-8 kb) of a Sau3A partial digest of genomic yeast DNA into BamHI-BgIII digested pRS202. pCG13 is pCG7 digested with EcoRI and religated. pCG14 is pCG7 digested with KpnI and religated. pCG15 is pCG7 digested with BamHI and religated. pCG16 is pCG7 digested with BgIII and religated. pCG17 is pCG7 digested with BgIII and BamHI and religated. pCG27 is the 2.2 kb BgIII-ClaI fragment of pCG16 cloned into BamHI/ClaI digested pRS202. pCG28 is the 1.1 kb Eco-RI-EagI fragment of pCG27 cloned into EcoRI-EagI digested pRS202. pCG31 is the 3.1 kb BgIII-SacI fragment of pCG16 cloned into BamHI-SacI digested pBSIIKS+ (Stratagene). pCG38 is the 2.6 kb HindIII fragment from pCG31 cloned into HindIII digested pRS202 in the same orientation as pCG31. pCG40 the 2.6 kb HindIII fragment from pCG31 cloned into HindIII digested pRS315. pCG41 is the 2.6 kb HindIII fragment from pCG31 cloned in to pRS305-2μ.

A precise deletion of PHD1 with the entire protein-coding region excised (phd1Δ1) was constructed using site-directed deletion mutagenesis (Kunkel, *et al.*, *Meth. Enzymol.*, *154*:367-382, (1987)). A 68 base synthetic

oligonucleotide was obtained with 5′-3′ has the 31 nt that immediately follow the PHD1 termiator, the hexanucleotide sequence recognized by the BgIII restriction endonuclease, and the 31 nt that immediately precede the ATG initiator codon of PHD1. This oligonucleotide was annealed to single-stranded pCG31 DNA prepared with helper phage M13K07 (Vieira, J. and Messing, J., *Meth. Enzymol.*, *153*:3-11, (1987)) in the dut$^-$, unt$^-$ *Escherichia coli* host, RZ1032 (Kunkel, *et al.*, *Meth. Enzymol.*, *154*:367-382,(1987)). After elongation, ligation, and transformation into a dut$^+$, ung$^+$ host, plasmid DNAs were screened for the absence of the 1.1 kb PHD1 coding sequence and the presence in its place of a unique new BgIII site diagnostic of a successful mutagenesis. PCG34 is one of these plasmids.

pCG36 is the 5 kb BgIII-BamHI fragment of PSE1076 (Elledge, S., unpublished data), a derivative of pNKY51 (Alani, *et al.*, *Genetics*, *116*:541-545, (1987)) which contains within its two hisG repeats the kan^r gene 3′ to the URA3 gene, cloned into BgIII digested pCG34 in the orientation EcoRI-BgIII-hisG-URA3-kan^r-hisG-BamHI/BgIII (both sites destroyed).

D. DNA Sequence Analysis of PHD1

The nucleotide sequence of the PHD1 gene, as shown in Figure 12, was determined by DNA sequence analysis of the 2.2 kb genomic BgIII-ClaI fragment of pCG7 that enhances pseudohyphal growth when present in pRS202 (pCG27). The sequence of the first 360 nucleotides of this fragment was determined on only one strand and is not shown. Restriction endonuclease fragments of this 2.2 kb fragment were subcloned into pRS202, double stranded DNA was prepared from those constructs by the method of (Haltiner, M., *et al.*, *Nucl. Acids Res.*, *13*:1015-1025, (1985)), and sequenced by

the dideoxy chain termination method of (Sanger, F.S., *et al.*, *Proc.Natl.Acad.Sci., USA*, 74:5463-5467, (1977)). Synthetic oligonucleotides designed from already sequenced portions of this DNA fragment were used to complete the sequencing of both of the strands of this fragment. One large open reading frame beginning 21 nucleotides 3′ of the EcoRI site and continuing through the ClaI site was found. Synthetic oligonucleotides were used to sequence the region around the ClaI site and a TAA termination codon was found 40 nucleotides 3′ of it. Termination codons were found in all 3 possible reading frames immediately upstream of the putative ATG and downstream of the terminator. Protein and DNA homology searches were performed at the National Center for Biotechnology Information using the BLAST network service (Altschul, S.F.,*et al.*, *J. Mol. Biol.*, *215*:403-410,(1990)).

E. Characterization of PHD1 Expression

1. Overexpression of PHD1 Inappropriately Activates Pseudohyphal Development

It was first examined whether PHD1 overexpression activated the pseudohypal pathway on media with abundant nitrogen sources. We streaked isogenic MATa/α strains that either did or did not overexpress PHD1 on SC plates lacking uracil (which contain ammonium sulfate, amino acids, and other nutrients) and minimal plates (which have high levels of ammonium sulfate as sole nitrogen source). After 24 hours microcolonies with enhanced pseudohyphal growth were observed on both of these media for the strain overexpressing PHD1 while the control strain made normal smooth symmetrical colonies. The pseudohyphae in the microcolonies were obscured by vegetative yeast cells soon after 24 hours, a phenomena which does not occur on SLAHD

medium presumably because the cells in the middle of the microcolony deplete their environment of nutrients and cannot grow rapidly. Furthermore, a strain that overexpresses PHD1, but not the isogeneic control strain, in appropriately activates the pseudohyphal pathway in liquid SC medium lacking uracil. A strain which is constitutively in the pseudohyphal mode, named CGH1, was also isolated; this strain grows as pseudohyphal microcolonies even in liquid rich medium.

2. PHD1 Overexpression Appears to Activate Pseudohyphal Growth by a Distinct Mechanism

Next is was investigated whether PHD1 overexpression enhances pseudohyphal growth in a manner similar to shr3 mutations which block efficient amino acid uptake (Gimeno, C.J., et al., (in press)). CG151 (an a/α strain which overexpressed PHD1) cannot grow on SPD plus 30 mM histidine indicating that overexpression of PHD1 does not phenocopy shr3 mutations (Ljungdahl, P.O., *et al.*, *Cell*, (1992)). Furthermore, the pseudohyphal growth of a/α Shr3⁻ strains resembles that of a/α wild-type strains when grown on SLAHD medium for 24 hours. By contrast, CG151 has greatly enhanced pseudohyphal growth after 24 hours on SLAHD. Like both Shr3⁻ and RAS2 ^{val19} a/α strains, CG151 has enhanced pseudohyphal growth on SPHD medium where 9.7 mM proline is sole nitrogen source. PHD1 overexpression appears to activate pseudohyphal growth by a mechanism distinct from mutation of SHR3.

The enhanced pseudohyphal growth caused by PHD1 overexpression was compared to RAS2 ^{val19}. On SLAHD medium PHD1 overexpression causes more dramatic pseudohyphal growth enhancement than RAS2^{val19} (data not shown). However, PHD1 overexpression does not confer heat shock sensitivity on

yeast cells while RAS2 ^{val19} does. The most simple explanation of this result is that PHD1 overexpression and RAS2 ^{val19} activate the pseudohyphal program by difference mechanisms.

3. Deletion of PHD1

Using standard genetic methods PHD1 has been deleted from the yeast genome. 10μg of pCG36 was subjected to a SalI-SacI double digest, extracted once with an equal volume of 1:1 mixture of phenol and 24:1 chloroform-isoamyl alcohol, extracted again with an equal volume of 24:1 chloroform-isoamyl alcohol, ethanol precipitated, and used to transform MB758-5B by the method of (Ito, H., *et al.*, *J. Bacteriol*, *153*:163-168, (1983)). Stable Ura$^+$ transformants were selected on SC plates lacking uracil. To confirm that the PHD1 gene has been deleted by homologous recombination, genomic DNA of the above transformants was prepared, digested with BamHI, and electrophoresed on a 0.7% agarose gel. The gel was processed for Southern blotting and probed with a ^{32}P labeled probe made from the 1 kb BgIII-EcoRI fragment of pCG16. In wid-type strains, this probe hybridizes to a 5.6 kb fragment and in strains with PHD1 deletions due to homologous recombination it hybridizes to a 9.6 kbfragment. The strain constructed in this fashion is CG238, and is viable when grown on rich medium.

F. Genetic and Physical Mapping of PHD1

A 1.1 kb EcoRI/ClaI restriction fragment containing most of the PHD1 coding sequence (Figure 12) was hybridized to the prime lambda-clone grid filters (a gift from L. Riles and M. Olson) that represent over 90% of the *S. cerevisiae* genome following their instructions.This probe hybridized to clone 4326 which contains a DNA fragment from the left arm

of chromosome XI. To confirm this map position, which is predicted to be near that of CDC16 and CEN11, PHD1 was mapped genetically. Strain CG343 (MATa ura3-52trp1::hisG cdc16-1 phd1Δ::URA3) was crossed by CG344 (MATα ura3-52 his4-619) to produce diploid CGX94 which was sporulated and subjected to tetrad analysis.Segregation of the two alleles of PHD1 in this cross was followed by scoring uracil prototrophy, the two alleles of CDC16 were followed by scoring growth at 36°C, and CEN11 was followed by scoring tryptophan prototrophy (trp1::hisG is tightly centromere linked). Analysis of 53 tetrads in which all five markers in this cross segregated in a Mendelian fashion revealed that phd1Δ::URA3 is 15.1 cM from cdc16-1 (37 parental ditypes, 0 nonparental ditypes, 16 tetratyupes) and that cdc16-1 is 19.8 cM from CEN11 (11 parental ditypes, 21 nonparental ditypes, 21 tetratypes). phd1Δ::URA3 is not linked to CEN11. Recombination frequencies were determined using the standard mapping functions: RF = (0.5x[TT-2NPD] + 4xNPD)/PD+NPD+TT for genetic linkage and (0.5xTT)/PD+NPD+TT for centromere linkage. Sequencing and restriction mapping experiments demonstrated that PHD1 is adjacent to the PR12 gene which has been mapped by hybridization to a chromosome-blot to chromosome XI (Foiani, M., *et al.*, *Mol. Cell. Biol.*, *9*:3081-3087, (1989)).

Thus, PHD1 was mapped physically and genetically to the left arm of chromosome XI and is adjacent to a gene encoding a DNA primase subunit. The PHD1 locus was defined by subcloning experiments pCG28 is notable because it shows that overexpression of sequences 5′ to PHD1 that presumably contain the promoter does not cause pseudohyphal growth enhancement. pCG27 shows that the carboxy-terminal 13 amino acids of PHD1 are dispensable for high copy pseudohyphal growth enhancement.

Because the amino acid sequences of many classes regulatory proteins are known, it is hypothesized that the DNA and predicted amino acid sequence of PHD1 can provide information about its function. PHD1 potentially encodes a 366 amino acid 40.6 kd polypeptide with a predicted isoelectric point of 9.0 (Figure 4A). PHD1 contains 9.8% strongly basic amino acids (K and R), and 8.5% strongly acidic amino acids (D and E) that cluster (26/36 basic and 19/31 acidic residues) in the carboxy-terminal 155 amino acids. Proline (21/34) and glutamine (13/19) residues cluster in the amino-terminal 145 amino acids and comprise 58%of residues 82-100. Some transcription factors have as their activation domains proline rich regions (Mitchell, P.J., and Rjian, R., *Science*, *245*:371-378, Mermod, N., *et al.*, *Cell*, *58*:741-753, (1989)).

Three proteins with significant homology to PHD1 were found: StuA (Miller, J.Y., *et al.*, *Genes Dev.*, *6*:1770-1782 (1992)), SWI4 (Andrews, B.J., and Herskowitz, I., *Nature*, *342*:830-833, (1989)), and cdc10^{+} (Aves, S.J., *et al.*, *EMBO J.*, *4*:457-463, (1985)). These three proteins are homologous to a domain of PHD1 mainly included in the carboxy-terminal charged region, are known or thought to be transcriptional regulatory proteins, and regulate the development of taxonomically diverse fungi (Miller, K.Y., *et al.*, *Genes Dev.*, *6*: 1770-1782, (1992); Andrews, B.J., and Herskowitz, I., *Nature*, *342*:830-833, (1989); Primig, M., *et al.*, *Nature*, *358*:593-597, (1992); and Lowndes, N.F., *et al.*, *Nature*, *355*:449-453 (1992)). Residues 184-289 of PHD1 are 70% identical and 84% similar to a region of StuA that partially coincides with one of the basic domains (residues 148-215) defined by (Miller, K.Y., *et al.*, *Genes Dev.*, *6*:1770-1782, (1992)). In addition, both PHD1 and StuA (Miller, K.Y., *et al.*, *Genes Dev.*, *6*:1770-1782, (1992)) have proline rich

amino-termini. Residues 188-275 of PHD1 are 27% identical and 53% similar to the DNA binding domain of SW14 which residues within amino acid residues 36-155 as defined by (Primig, M., *et al.*, *Nature*, *358*:593-597, (1992)). Residues 253-280 of PHD1 are 39% identical and 67% similar to cd10$^+$ in a region known to be similar to the SW14 DNA binding domain (Primig, M., *et al.*, *Nature*, *358*:593-597, (1992)).These homologies suggest that PHD1 is a DNA binding transcription factor and are consistent with our genetic results which suggest that PHD1 is a regulatory protein which controls pseudohyphal growth. Interestingly, StuA regulates pseudohyphal growth (Primary and secondary sterigmata formation) during conidiophore morphogenesis in *A. nidulans* (Miller, K.Y., *et al.*, *Genes Dev.* *6*:1770-1782, (1992)).

G. PHD1 Protein Localization

An epitope tagged (Kolodziej, P. A., and Young, R. A., *Meth. Enzymol.*, *154*:367-382, (1991)) PHD1 that is being overexpressed and is activating PHG immunolocalizes to the nucleus. This supports the idea that PHD1 is a transcriptional regulatory protein. Epitope tagging of PHD1 was performed as described by (Kolodziej, P.A., and Young, R.A., *Meth. Enzymol.*, *154*:367-382, (1991)) using site-directed insertion mutagenesis (Kunket, T. A., *et al*, *Meth. Enzymol*, *154*:367-382, (1987)). A9 amino acid epitope from the influenza virus hemagglutinin protein HA1 (Wilson, I. A., *et al*, *Cell*, *37*:767-778, (1984)) was introduced into the PHD1 sequence between amino acid residues 355 and 356 (PHD1::FLU1). This position was chosen because amino acid residues 354-366 are not required for high copy PHD1 enhancement of pseudohyphal growth (pCG27 in Figure 3B). A

synthetic oligonucleotide was synthesized with 27 nt encoding the HA1 epitope (in the noncoding orientation) flanked on the 5′ side with 17 bases and on the 3′ side with 19 bases complementary to the PHD1 sequence. As in the construction of phd1Δ1, the oligonucleotide was annealed to single-stranded pCG31 DNA. After elongation, ligation, and transformation into a dut$^+$ unt$^+$ host, plasmid DNAs were screended for the presence of a new AatII restriction site diagnostic for successful mutagenesis. pCG35 is one of these plasmids. pCG37 is the 2.6 kb HindIII fragment from pCG35 cloned into HindIII digested pRS202 in the same orientation as in pCG31. pCG37 which contains PHD1::FLU1 ehances pseudohyphal growth to the same degree as pCG38.

EXAMPLE 7 Isolation of Pseudohyphal Genes from Candida Albicans

Methods identical to those used in Example 6 to identify pseudolyphal genes in *S. cerevisiae* were also used to identify pseudohyphol genes in *C. albicans*. *S. cerevisiae* strain CGX69 was transformed with a genomic *C. albicans* library constructed from a Sau3A partial digest of strain 1006 (Arg$^-$ Ser$^-$ Lys$^-$ ura3 Mpa^R) (Sikorski, R.S. and P. Hieter, Genetics 122: 19-27 (1989)) the URA3 marked high copy vector pRS202, a modification of pRS306 (Sikorski, R.S. and P. Hieter, Genetics 122: 19-27 (1989)). The yeast was plated on SC plates lacking uracil. 200,000 colonies were generated, 40% of which contained plasmid inserts; of these, more than 90% contained inserts greater than 4 Kb in length. Colonies were screened by washing the plates to remove cells and then examining the plates for colonies imbedded in the agar. Of the imbedded colonies, those displaying pseudohyphal growth were selected. From these colonies, three *C. albicans* genes were isolated. The first gene,

designated CPH1, was found four times; CPH2 was found three times; and CPH3 found once. All three genes were retransformed into CGX69 to confirm that they enhanced pseudohyphal growth. The results of retransformation indicated that the genes increased pseudohyphal growth both in liquid medium and in regular yeast medium (nitrogen base with mixed amino acids and 2% glucose (Difco)). CPH1 has been sequenced, as shown in Figure 14. CPH1 was found to be homologous to the yeast gene STE12, which is a transcriptional factor.

Another gene, PHD5, was isolated from wild-type *C. albicans* in a similar manner. *S. cerevisiae* strain CGX68 was transfected with a *C. albicans* genomic library constructed in a CEN Ura$^+$ vector. The transfected cells were plated on low ammonium SLAHD plates, and subsequently screened as above for those colonies demonstrating pseudohyphae formation. The gene designated PHD5 was isolated twice from these colonies. Retransformation and linkage analysis confirmed the relation between the PHD5 gene and the pseudohyphal phenotype. The PHD5 gene has been sequenced, as shown in Figure 15. No homology to CPH1 has been found.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. An isolated fungal dimorphism gene.

2. An isolated fungal dimorphism gene of Claim 1 which is a yeast dimorphism gene.

3. An isolated yeast dimorphism gene of Claim 2 which is a Saccharomyces cerevisiae gene or a Candida albicans gene.

4. An isolated dimorphism gene of yeast origin having DNA sequence selected from the group consisting of:
   a) the DNA sequence of Figure 12;
   b) the DNA sequence of Figure 14;
   c) the DNA sequence of Figure 15; and
   d) DNA sequence which hybridize to all or a portion of the DNA sequences of a), b), or c).

5. An isolated protein encoded by a fungal dimorphism gene.

6. An isolated protein of Claim 5 encoded by a yeast dimorphism gene.

7. An isolated protein encoded by a yeast dimorphism gene selected from the group consisting of PHD1, CPH1, and PHD5, and DNA which hybridizes to all or a portion of PHD1, CPH1 or PDH5.

8. An isolated protein of Claim 7 wherein PHD1 has the sequence of Figure 12; CPH1 has the sequence of Figure 14; and PHD5 has the sequence of Figure 15.

9. A recombinantly produced protein encoded by a fungal dimorphism gene.

10. A recombinantly produced protein of Claim 9 wherein the fungal dimorphism gene is a yeast dimorphism gene.

11. A recombinantly produced protein encoded by a yeast dimorphism gene, the yeast dimorphism gene selected from the group consisting of PHD1, CPH1, and PHD5, and genes which hybridize to all or a portion of PHD1, CPH1, or PHD5.

12. A hybridization probe derived from a yeast dimorphism gene.

13. A hybridization probe of Claim 12, derived from PHD1, CPH1, or PHD5.

14. A pair of PCR primers derived from a yeast dimorphism gene.

15. A pair of PCR primers of Claim 14, derived from PHD1, CPH1, or PHD5.

16. A method of isolating, from a dimorphic fungus, a dimorphism gene which is an activator of dimorphism in the dimorphic fungus, comprising the steps of:

a) producing a plasmid genomic DNA library from a dimorphic fungus;

b) transforming a dimorphic Mata/α strain of Saccharomyces cerevisiae which has an auxotrophic marker with the plasmid genomic DNA library, thereby producing a mixture of yeast transformed cells; and untransformed yeast cells;

c) culturing the mixture on medium containing an appropriate selective agent for an appropriate length of time for untransformed yeast cells to die, thereby producing colonies of transformed dimorphic yeast cells which contain genomic DNA from the dimorphic fungus, present in a plasmid;

d) plating colonies produced in (c) at a density of 2,000 colony forming units per plate on medium appropriate for growth of the colonies, thereby producing plated colonies;

e) culturing the plated colonies for sufficient time for transformed yeast cells in which a dimorphism gene is overexpressed to display a fuzzy morphology, thereby producing colonies displaying a fuzzy morphology;

f) selecting colonies displaying a fuzzy morphology, which is indicative of pseudohyphal growth; and

g) isolating genomic DNA from plasmids in transformed cells in the selected colonies,

wherein the isolated genomic DNA is a dimorphism gene.

17. The method of Claim 16 wherein the dimorphic fungus is a yeast.

18. The method of Claim 17, wherein the yeast is Saccharomyces or Candida.

19. The method of Claim 16, in which the plasmid library comprises genomic DNA from the fungus of interest inserted in a Saccharomyces cerevisiae vector with the 2μ origin of replication.

20. A method of isolating, from a dimorphic fungus, DNA which encodes an activator of dimorphism in the dimorphic fungus, comprising the steps of:

a) producing a plasmid cDNA library from a dimorphic fungus;

b) transforming a dimorphic Mata/α strain of Saccharomyces cerevisiae which has an auxotrophic marker with the plasmid cDNA library, thereby producing a mixture of yeast transformed cells; and untransformed yeast cells;

c) culturing the mixture on medium containing an appropriate selective agent for an appropriate length of time for untransformed yeast cells to die, thereby producing colonies of transformed dimorphic yeast cells which contain genomic DNA from the dimorphic fungus, present in a plasmid;

d) plating colonies produced in (c) at a density of 2,000 colony forming units per plate on medium appropriate for growth of the colonies, thereby producing plated colonies;

e) culturing the plated colonies for sufficient time for transformed yeast cells in which a dimorphism gene is overexpressed to display a fuzzy morphology, thereby producing colonies displaying a fuzzy morphology;

f) selecting colonies displaying a fuzzy morphology, which is indicative of pseudohyphal growth; and

g) isolating cDNA from plasmids in transformed cells in the selected colonies,

wherein the isolated cDNA encodes an activator of dimorphism.

21. The method of Claim 20 wherein the dimorphic fungus is a yeast.

22. The method of Claim 21, wherein the yeast is Saccharomyces or Candida.

23. The method of Claim 21, in which the plasmid library comprises cDNA from the fungus of interest inserted in a Saccharomyces cerevisiae vector with a galactose inducible promoter.

24. A method of isolating, from a dimorphic fungus, a dimorphism gene which is a suppressor of dimorphism in the dimorphic fungus, comprising the steps of:

a) producing a plasmid genomic DNA library from a dimorphic fungus;

b) transforming a dimorphic Mata/α strain of Saccharomyces cerevisiae which has an auxotrophic marker with the plasmid genomic DNA library, thereby producing a mixture of yeast transformed cells; and untransformed yeast cells;

c) culturing the mixture on medium containing an appropriate selective agent for an appropriate length of time for untransformed yeast cells to die, thereby producing colonies of transformed

dimorphic yeast cells which contain genomic DNA from the dimorphic fungus, present in a plasmid;

d) plating colonies produced in (c) at a density of 200 colony forming units per plate on medium appropriate for growth of the colonies, thereby producing plated colonies;

e) culturing the plated colonies for sufficient time for transformed yeast cells in which a dimorphism gene is suppressed to display a round and symmetrical morphology, thereby producing colonies displaying a round and symmetrical morphology;

f) selecting colonies displaying a round and symmetrical morphology, which is indicative of suppressed pseudohyphal growth; and

g) isolating genomic DNA from plasmids in transformed cells in the selected colonies,

wherein the isolated genomic DNA is a dimorphism gene.

25. The method of Claim 24 wherein the dimorphic fungus is a yeast.

26. The method of Claim 25, wherein the yeast is Saccharomyces or Candida.

27. The method of Claim 24, in which the plasmid library comprises genomic DNA from the fungus of interest inserted in a Saccharomyces cerevisiae vector with the 2μ origin of replication.

28. A method of isolating, from a dimorphic fungus, DNA which encodes a suppressor of dimorphism in the dimorphic fungus, comprising the steps of:

a) producing a plasmid cDNA library from a dimorphic fungus;

b) transforming a dimorphic Mata/α strain of Saccharomyces cerevisiae which has an auxotrophic marker with the plasmid cDNA library, thereby producing a mixture of yeast transformed cells; and untransformed yeast cells;

c) culturing the mixture on medium containing an appropriate selective agent for an appropriate length of time for untransformed yeast cells to die, thereby producing colonies of transformed dimorphic yeast cells which contain genomic DNA from the dimorphic fungus, present in a plasmid;

d) plating colonies produced in (c) at a density of 200 colony forming units per plate on medium appropriate for growth of the colonies, thereby producing plated colonies;

e) culturing the plated colonies for sufficient time for transformed yeast cells in which a dimorphism gene is suppressed to display a round and symmetrical morphology, thereby producing colonies displaying a round and symmetrical morphology;

f) selecting colonies displaying a round and symmetrical morphology, which is indicative of suppressed pseudohyphal growth; and

g) isolating cDNA from plasmids in transformed cells in the selected colonies,

wherein the isolated cDNA encodes an activator of dimorphism.

29. The method of Claim 28 wherein the dimorphic fungus is a yeast.

30. The method of Claim 29, wherein the yeast is Saccharomyces or Candida.

31. The method of Claim 29, in which the plasmid library comprises cDNA from the fungus of interest inserted in a Saccharomyces cerevisiae vector with a galactose inducible promoter.

32. A method of identifying a fungal dimorphism gene, comprising screening a DNA library from the fungus of interest with a hybridization probe derived from the coding sequence of a yeast dimorphism gene selected from the group consisting of: PHD1, CPH1, and PHD5, under conditions appropriate for hybridization of the probes to complementary sequences.

33. A Saccharomyces cerevisiae strain which is deleted for the gene PHD1.

34. A Candida albicans strain which is deleted for the gene CPH1 or the gene PHD5.

35. A yeast strain which constitutively undergoes pseudohyphal growth.

36. A Saccharomyces cerevisiae strain which constitutively overexpresses a dimorphism gene and undergoes pseudohyphal growth.

37. A Saccharomyces cerevisiae strain of Claim 36 which constitutively overexpresses PHD1.

38. A mutant yeast strain in which a gene encoding a permease required for amino acid transport is mutated and amino acid transport is impaired.

39. The mutant yeast strain of Claim 38 which is a mutant Saccharomyces cerevisiae in which the SHR3 gene is mutated.

40. An isolated gene of yeast origin which encodes an endoplasmic reticulum protein required for amino acid transport in yeast.

41. The isolated gene of Claim 40 which is of Saccharomyces cerevisiae origin.

42. The isolated gene of Claim 40 which has the DNA sequence of Figure 5.

43. A 23.5 kDa yeast membrane protein encoded by the SHR3 gene having the DNA sequence of Figure 5.

44. A method of altering dimorphic change in a yeast cell, comprising introducing into the yeast cell an agent which interferes with a gene which affects the dimorphic change or with a product of said gene.

45. The method of Claim 44 wherein the agent is selected from the group consisting of: peptides, anti-sense nucleotides, small inorganic molecules and small organic molecules.

46. The method of Claim 44 wherein the gene is selected from the group consisting of: PHD1, CPH1 and PHD5.

FIG. IA

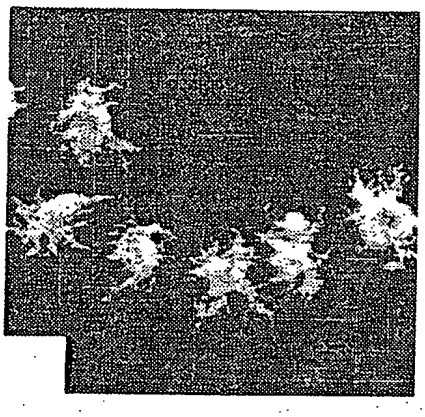


FIG. IB

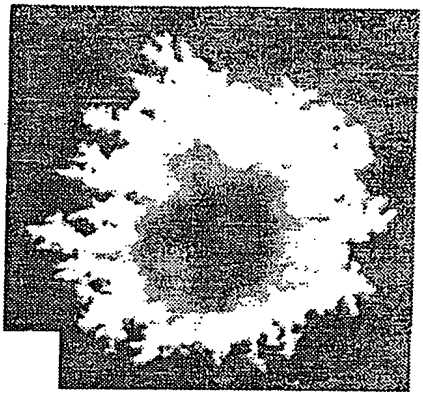


FIG. IC

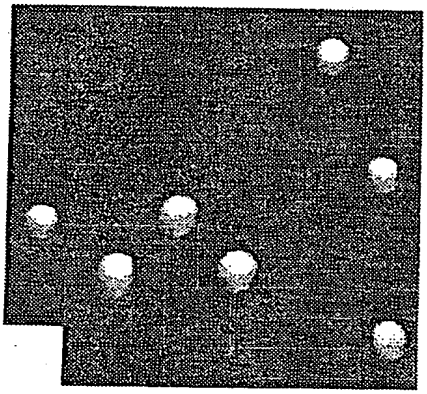


FIG. ID

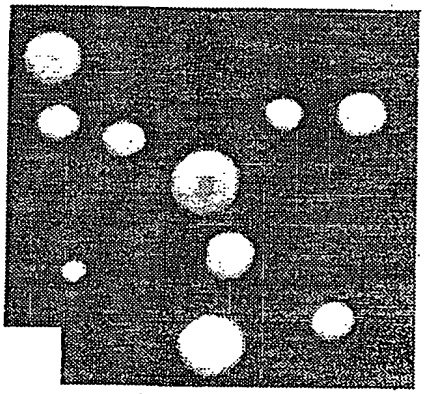


FIG. IE

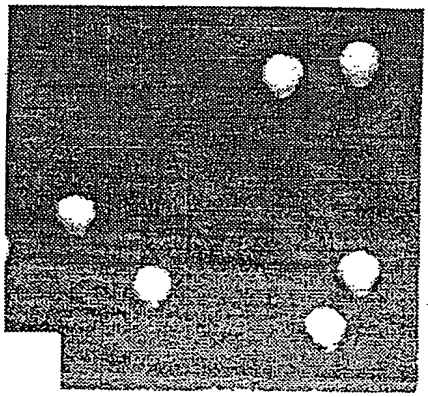


FIG. IF

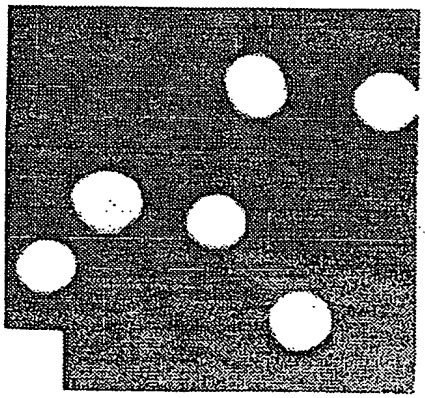


FIG. 2B

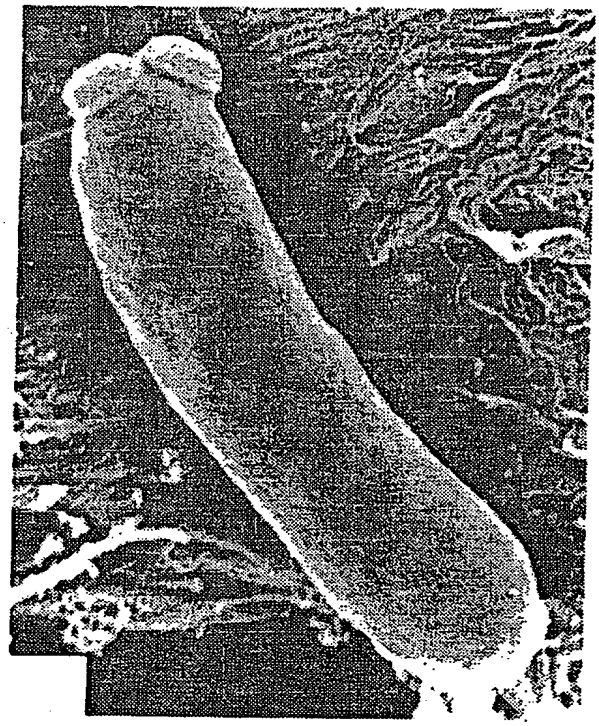


FIG. 2A

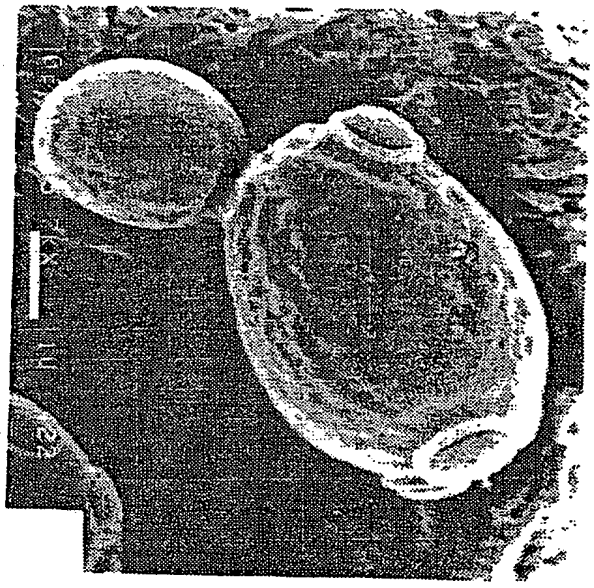


FIG. 3B

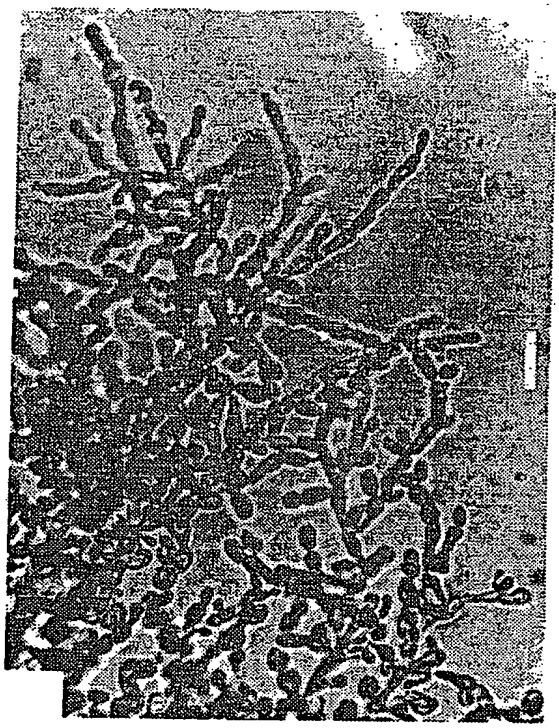
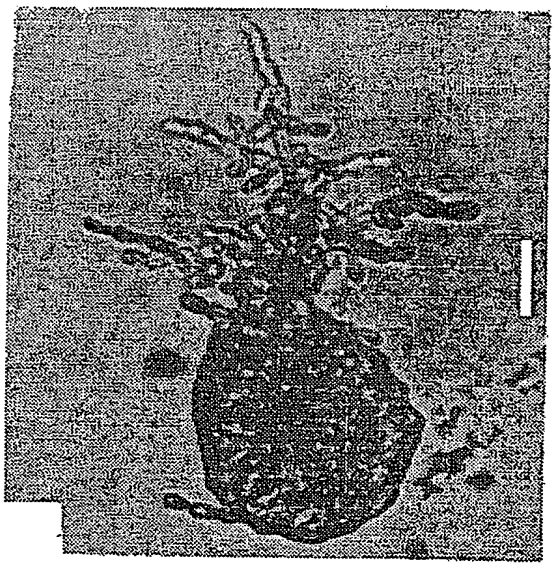


FIG. 3A



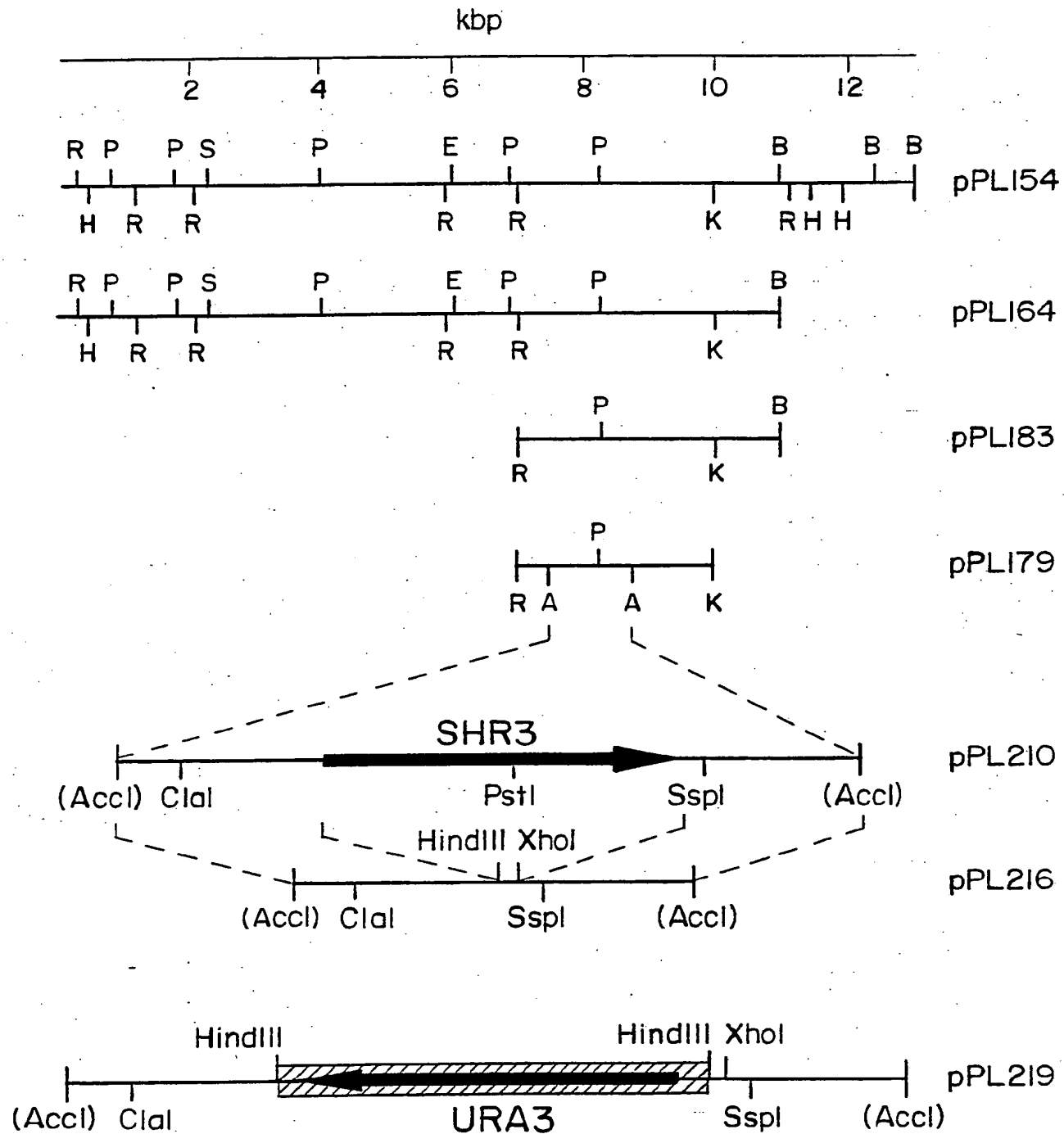


FIG. 4

SHR3

```
   1  GTAGACGTCTTCAATCTCTATCCTGTCCTCTACATTAGATTACTTTCTCATTTTCTCAGA   60
  61  AAACCTTTTAAGCTCATCGATTAAGAAGAAAAAAATCTTAGAAAATTTTCAGGAATGCCG  120
 121  GGTAAACGTATGGGGAAAGGACTTGGTGAATCGAGTTAATGTGGAGTTCGTAATAATAGT  180
 181  ACTCTCATACCAAGTTTTCCATCCATAATTGTTTTTGGTTGCCGTTACTTTACACCATCA  240
 241  CCTTTCGCCAATGTAATTGGTGGCTCTTTCCCTCTCCATCTTTTTTTATAATGTACTAAA  300
 301  ATTAATACACGATAACAATTTTTTCGGGTTGTGTAAAAACTCTGAAAAATATAAAAAGTT  360
 361  GATGAAAGGACAGATTTAAGTATTTTGGAAGACAGCCGTAAATTCATTGGACTACAGAGC  420
 421  ATAAAATCCCGTGATACGATAAAACTCTAACGATGTTCTCATATTCAGATTTCTGTTCTA  480
                                   M  F  S  Y  S  D  F  C  S  I
 481  TCGGTACGGCCATGATCTTATCGGCCACAACGTTCCTAATGGGTGTTTTCTTCAGTAACA  540
        G  T  A  M  I  L  S  A  T  T  F  L  M  G  V  F  F  S  N  M
 541  TGCCATACGATTATCATCTTTTATTTAATCCTAACTCTACTCAAGAGCATTTCGATTTGG  600
        P  Y  D  Y  H  L  L  F  N  P  N  S  T  Q  E  H  F  D  L  A
 601  CACTGAGACATTACCAAATTTTACATGAGACTCCATTGCCGGTGATTGTTACTTTGTGCG  660
        L  R  H  Y  Q  I  L  H  E  T  P  L  P  V  I  V  T  L  C  V
 661  TTGTTGCCGGTATTGGCTTAGTTGGTGGTACAATTAAGGTTTTCAAGCCAAACCCTGAAC  720
        V  A  G  I  G  L  V  G  G  T  I  K  V  F  K  P  N  P  E  L
 721  TGCAGATGTTTGAGTATTGTTCATTGGGGTTGTACGTGTTGGCTATCTGTGTGTTTCTCA  780
        Q  M  F  E  Y  C  S  L  G  L  Y  V  L  A  I  C  V  F  L  T
 781  CTAATGTGAAAACAGGTATCGACTGTTCTGTGAGCCATAATTGGGAGAAGTTACGAAATC  840
        N  V  K  T  G  I  D  C  S  V  S  H  N  W  E  K  L  R  N  Q
 841  AGGGTTTGGCAGTTATTGCTTCCTCCAACATAATTTTATTAGTTATGTTTGCCGGTGTTA  900
        G  L  A  V  I  A  S  S  N  I  I  L  L  V  M  F  A  G  V  I
 901  TCATCCTACAAATTGGTTTGTGGTACAGTAACTGGGATTTGCAAAAAAGATTAAAGGAGT  960
        I  L  Q  I  G  L  W  Y  S  N  W  D  L  Q  K  R  L  K  E  F
 961  TTTATGCTCAAGAAGAAAGAGAAGCTGCCAATGCCGGTAAAAAGACTGAGAAAGTTGACA 1020
        Y  A  Q  E  E  R  E  A  A  N  A  G  K  K  T  E  K  V  D  N
1021  ATGCGAAAAAGAATGATAACAAATCTAAAGGTGCTCAAAAGAGGAAGAACGCCAAAAAAT 1080
        A  K  K  N  D  N  K  S  K  G  A  Q  K  R  K  N  A  K  K
1081  AGGCCATAATATTTTTAACATTTTCTCTTTTTGTCCCTCTTTTCCCGTCTGTGACGAAAC 1140
1141  AGACGATTATACGAATCTAGTTGTATAGTAAGATCAATATTTATTATATAAATACTGTTG 1200
1201  AATAAATAAAACTCTATCTGCTTCGTGCATAAATTTACAAATAATTCCAAAGAAATAGCA 1260
1261  ACAAGTACCAGCCTTGTTTAGAAGTACGAAAATCTTGGAATCCACTTTTTTCTATTTTTA 1320
1321  AATAATTTCTCTCAAAAAGCTATTGGTTTGCTGAATATTTGGCCGAGTAATAGTAGAC  1378
```

Figure 5

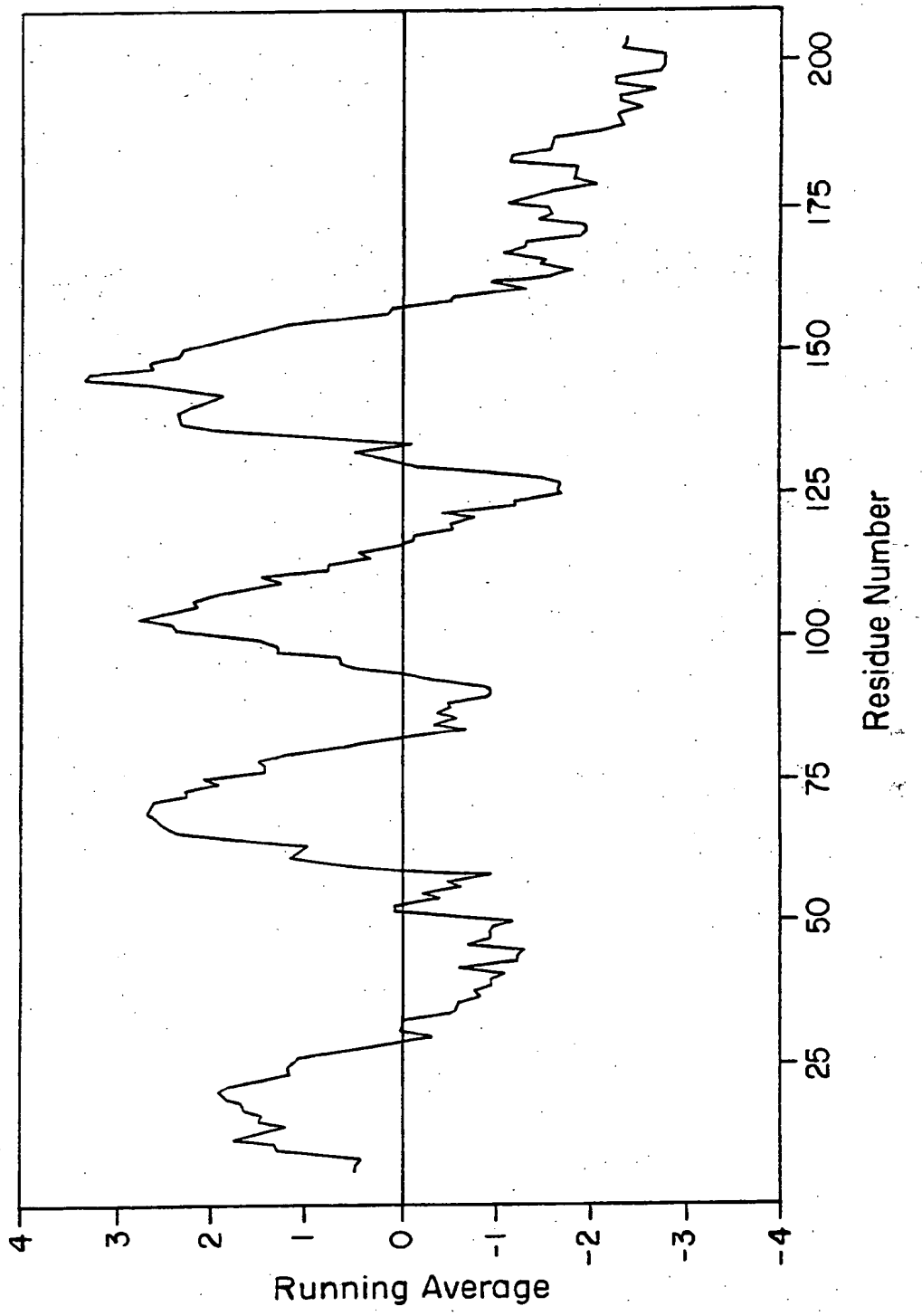


FIG. 6

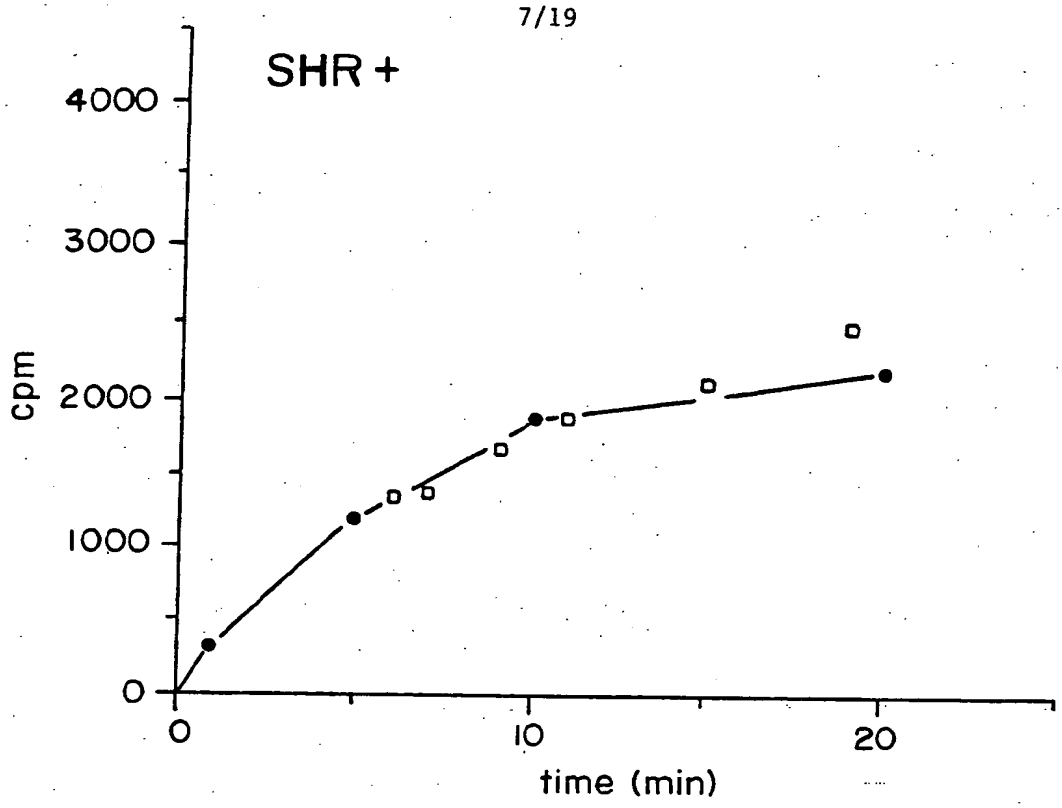


FIG. 7A

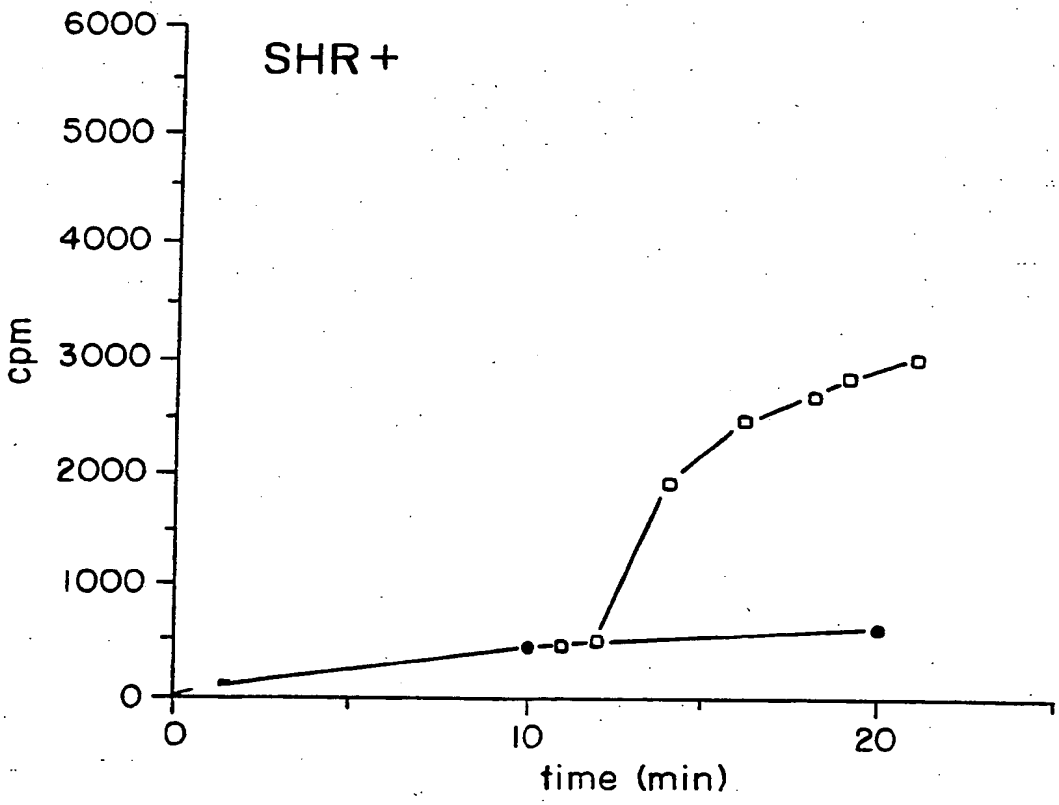


FIG. 7B

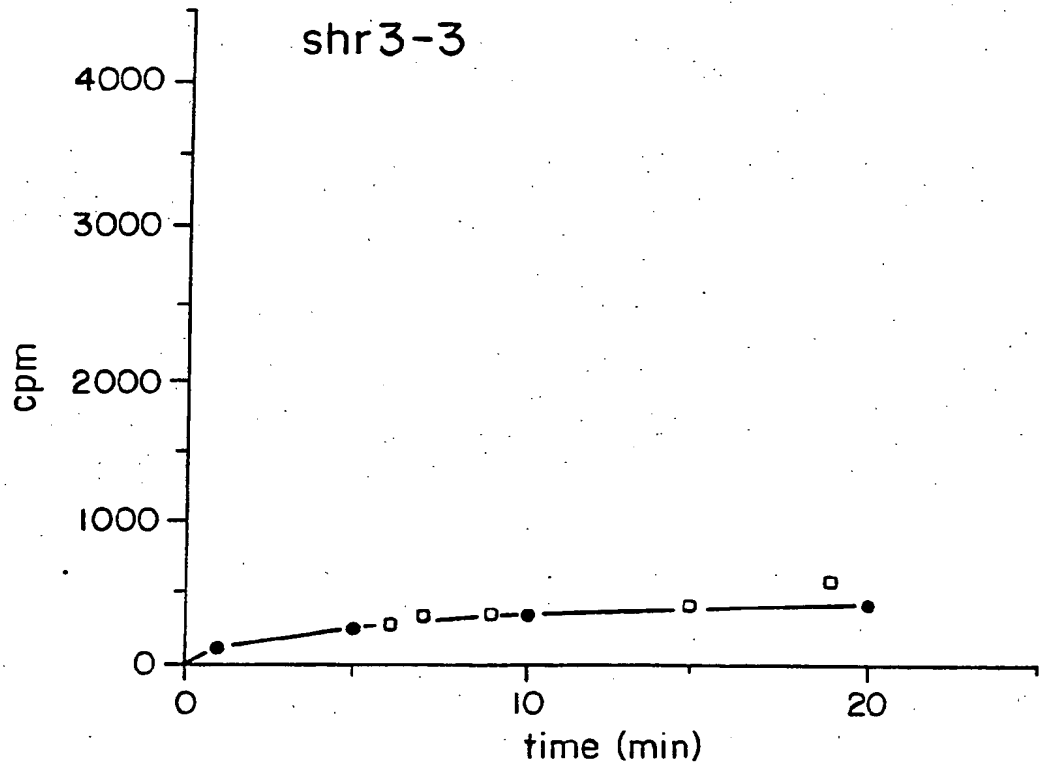


FIG. 7C

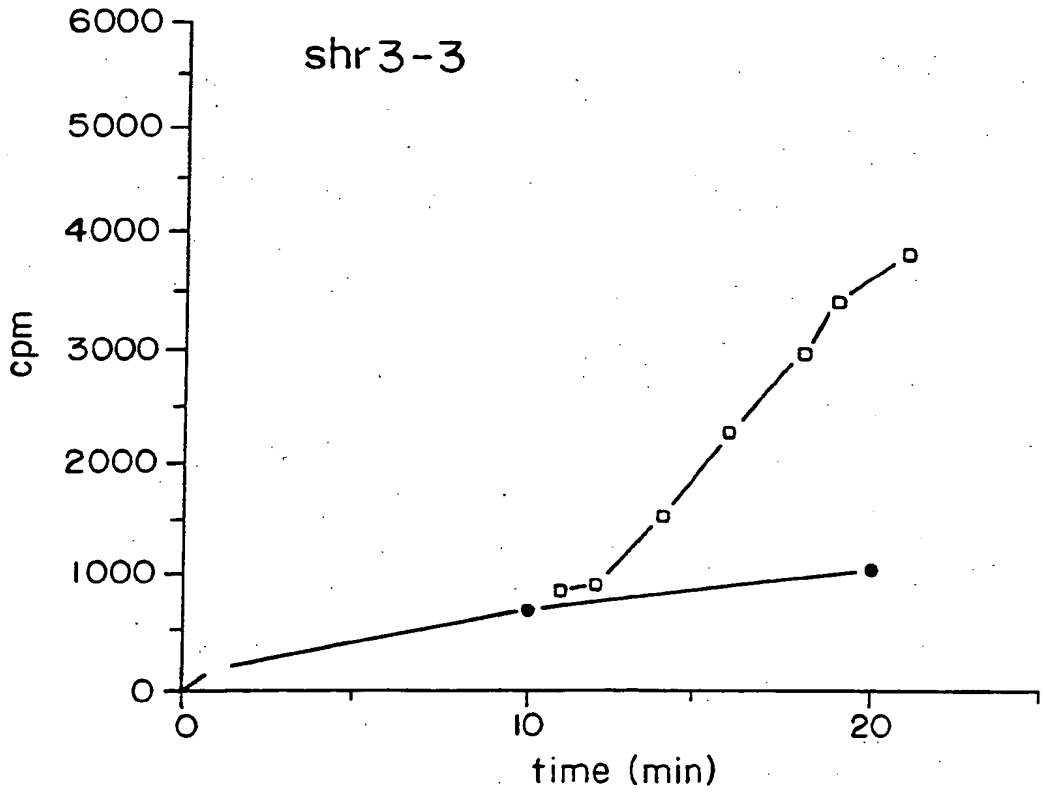


FIG. 7D

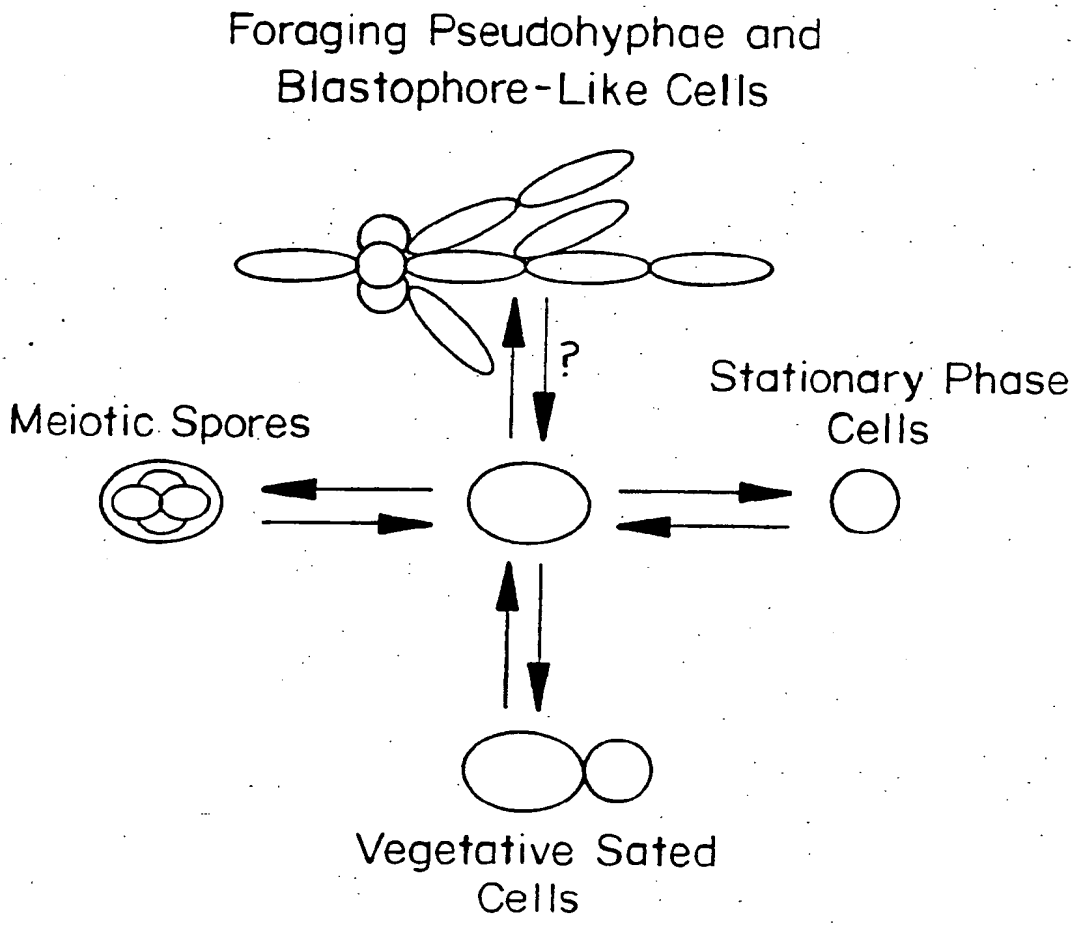


FIG. 8

FIG. 9A

FIG. 9B

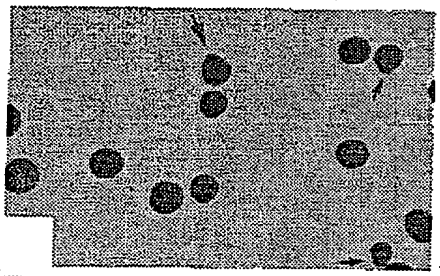


FIG. 9C

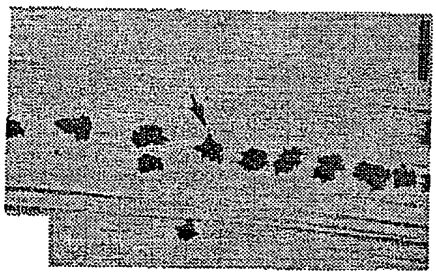


FIG. 9D

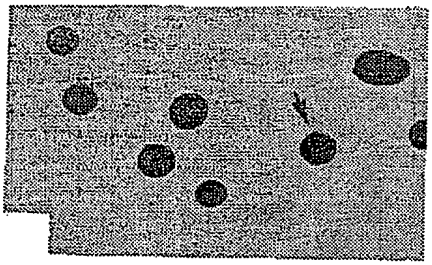


FIG. 9E

FIG. 9F

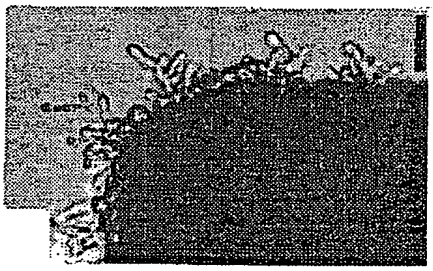


FIG. 9G

FIG. 9H

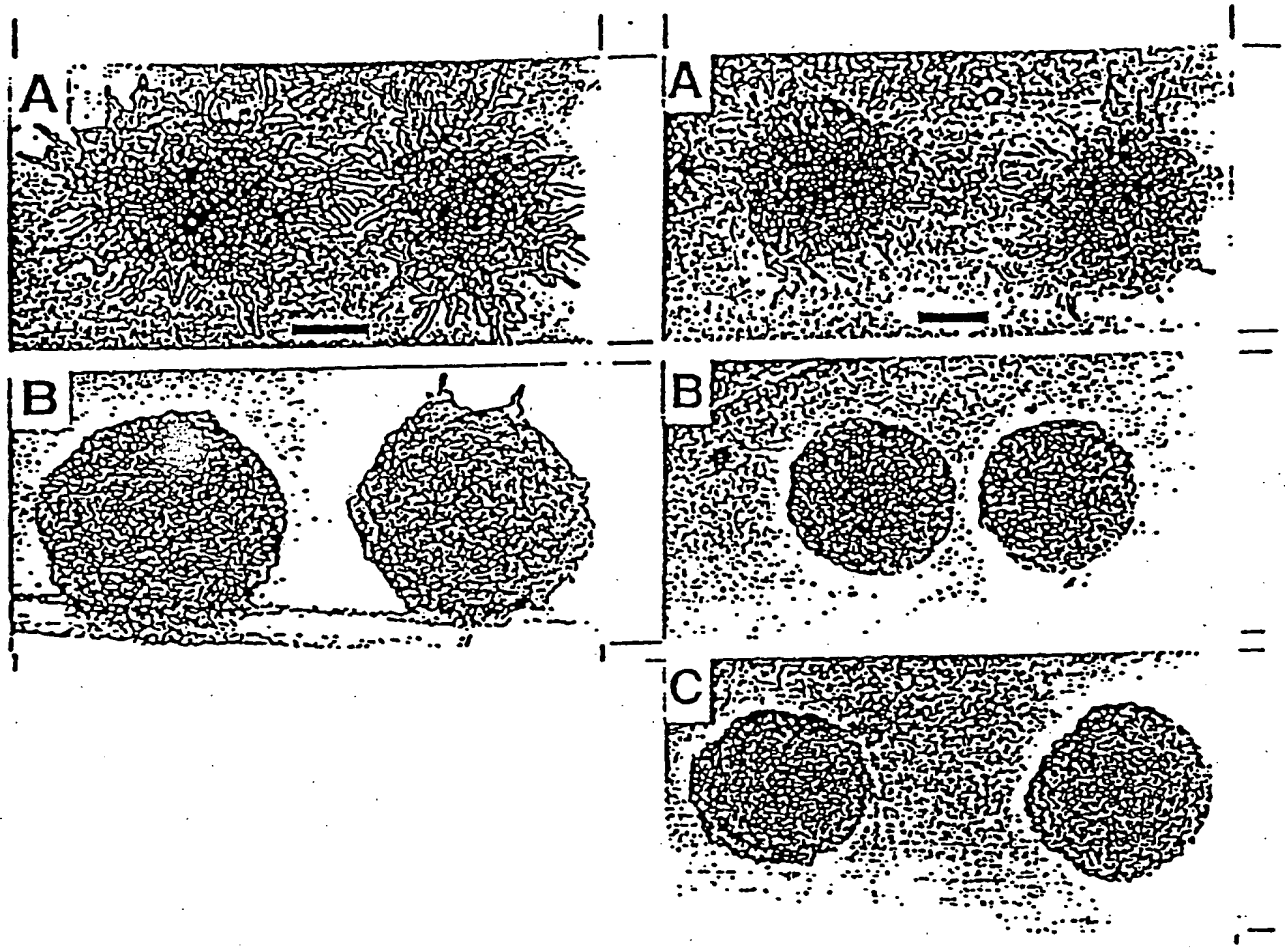


Figure 10

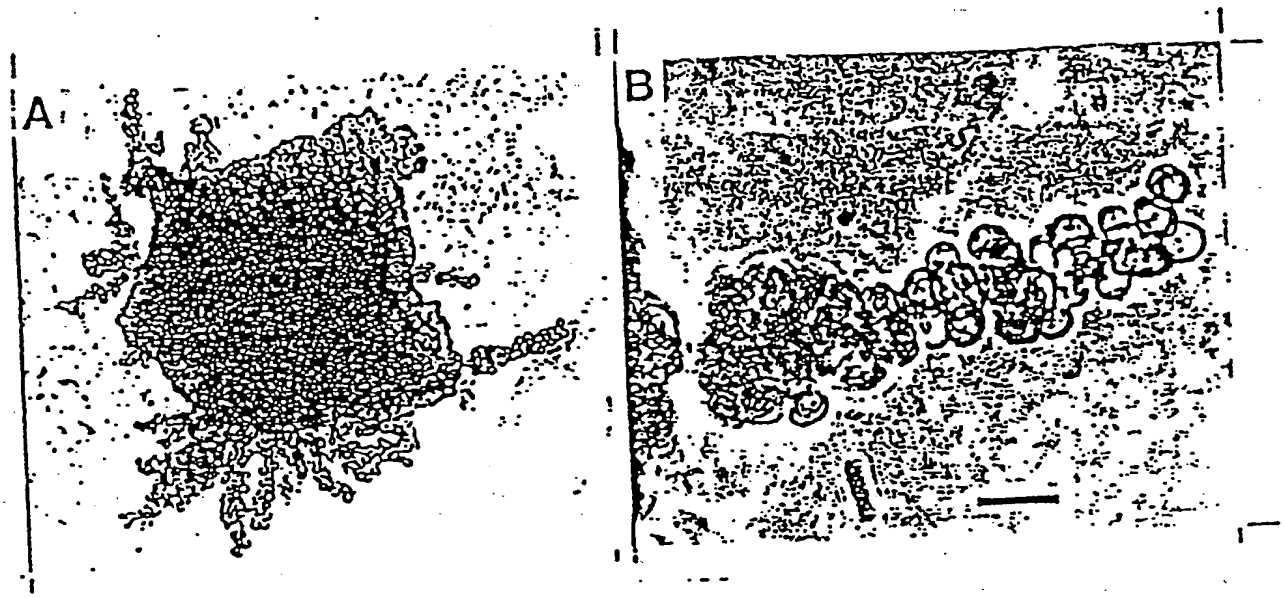


Figure 11

FIG. IIB

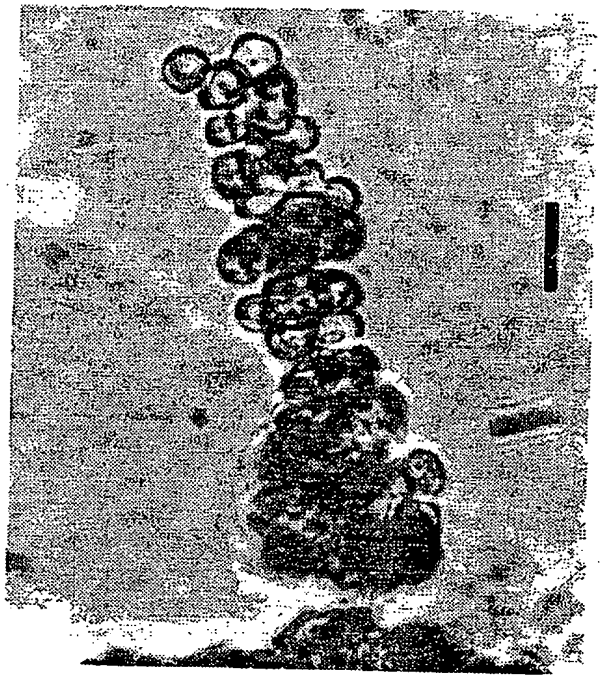


FIG. IIA

FIGURE 12A

FIGURE 12B

```
PHD1 184 KPRVITDKEDENTICYQVEANGISVVRRADNNMINGTKLLNVTKMTRGRRDGILRSE
stuA 127 ——TA-L———CSL———K-VC-A—E—C———————JG———————K——
SWI4  35  -A-YS-TDVYE—IRGFKTKI-K—TKQDW—I-QVFKQAQFSKIK-TK—EK-SND
cdc10 13
```

```
KVREVVKIGSMHLKGVWIPFERAYILAQREQILDHLYPLFVKDIESIV 289 PHD1
——N———P———————D—LEF-NK-K-T-L———————QH-SKIL 232 stuA
MQH-K-QG-YGRFQ-T——LDS-KF-VNKYK-I-           129 SWI4
          QID——V-YD——ISI-K-YGVYKI-Q—         161 cdc10
```

FIGURE 12C

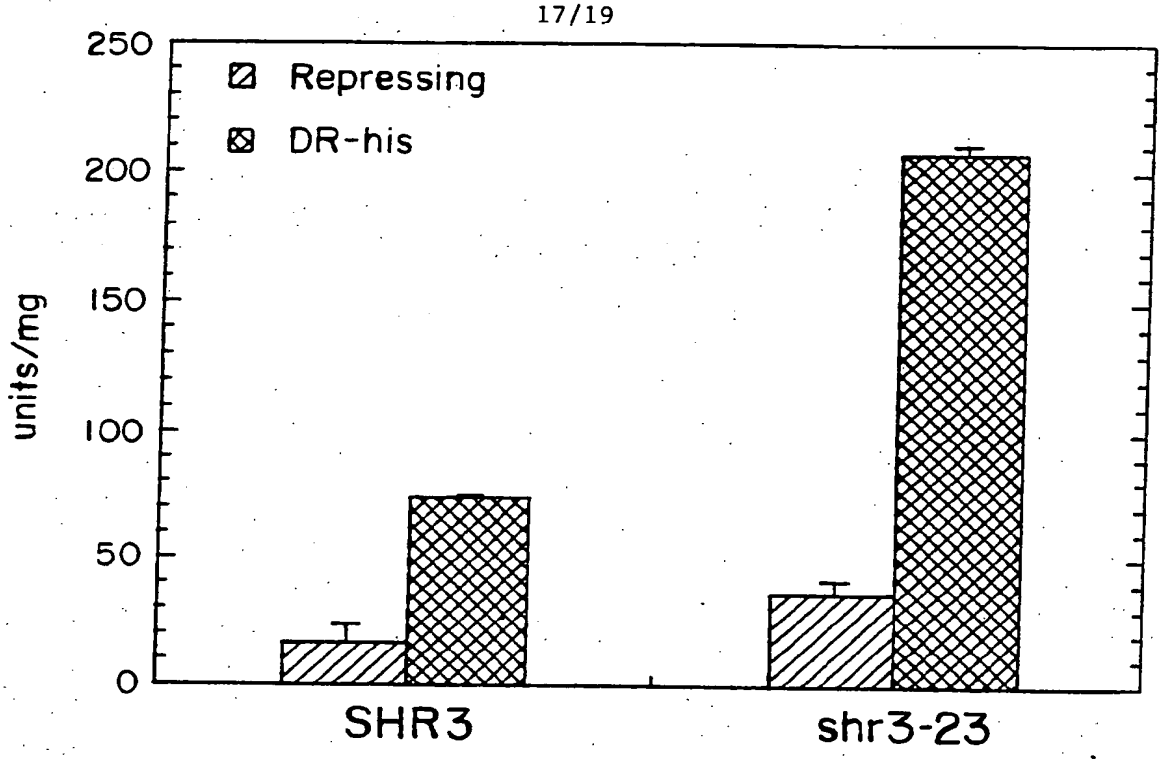


FIG. 13A

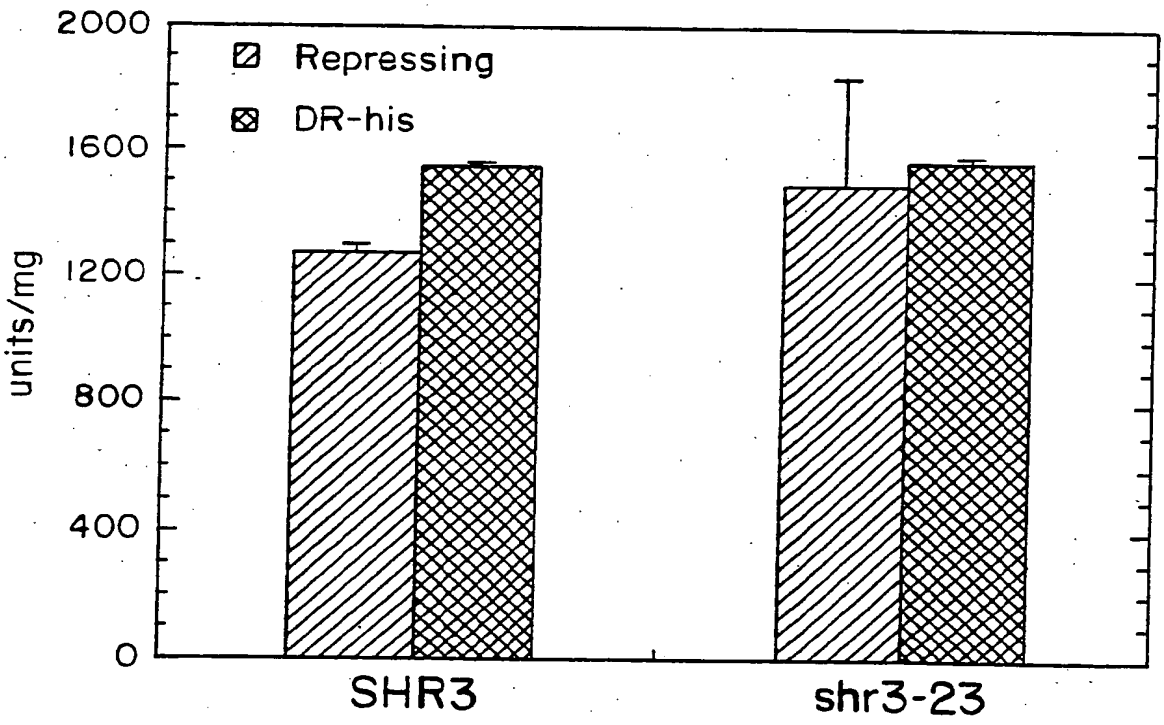


FIG. 13B

FIGURE 14

CATTAATTCTTAGTTAAATATATTTCTTTTCACTTTTTTCACTTTTTTCATTTT
TCAATTGTTGTGCCCCCCCCTCGTTGCGGATCAAATAATATAACTGCCTAA
TACACTCTTTCGCCATGTCAATTACTAAAACATACAATGGTGATCCTACAT
CACTTGTACCAACTCAACTGGTAAAAGAATCATTAAGACTAATTGAAGAT
TTAAAATTCTTTTTAGCCACTGCTCCAGCAAATTGGCAAGAGAATCAAGT
GATTCGAAGATACTATTTAAACCACGATGAAGGTTTTGTCAGTTGTGTTTA
TTGGAATAACTTATATTTTATCACTGGTACCGATATCGTCCGTTGTATCGTT
TATAAATTTGAACATTTTGGAAGGAAAATCATTGATCGGAAAAAGTTTGA
AGAAGGTATTTTCTCCGATTTAAGAAATTTGAAATGTGGCGCCGATGCAA
TTTTGGAACCTCCTCGTTCAGAGTTTTTAGAATTCTTATTTAAAAATTCTTG
TTTGAGAACACAAAAGAAACAAAAAGTGTTTTTTTGGTTTAATGTTCCTCA
TGATAAGTTAATGGCAGATGCCTTGGAACGAGATTTGAAAAAGGAAAAA
ATGGGTCAAAGACCAACAACAATGGCCCATCGAGAACCAGCATTATCAT
TCCATTACGACGAGTCTTCCAGTTTATACACTCAATTGGGTAAACACATGG
AAACTCAGAAGAGAATCAACGACGCAGCCACAAGCTCTACTTCTAATACT
GCTACCACTTTAACCGACACCGGTGTATCCTCAGGATTGAATAATACCAC
TTCTGGTGGCGGCAGTGATAGTGCAACCTCCACACACAACAACAATGAG
GCATCGACCAAACCAAGTAATGGCAGTGAAAAATCGTCACCGGAGTACA
CTACAACTGCCCGCGGTAGAGATGAGTTTGGATTCCTTAATGAAGCCACA
CCAAGTCAATACAAAGCCAATTCAGATTATGAAGACGATTTCCCATTGGA
TTATATCAATCAGACCACTCAAAATTCTGAAGATTATATTACTTTGGATGC
AAATTATCAGGCAGGAAGTTATGCAAATATGATCGAAGACAATTACGATT
CATTTTTGGATGCAACACTATTTATACCTCCAAGTCTTGGCGTACCTACAG
GTACAGCTGCGACTGCAACAACATCAAACCAAGTTGCCTTCAACGACGA
ATACTTGATTGAACAAGCCCAACCAATAAGGACTCCACTACCCCCAATAT
CATCATCAACAATATCCGGATTATTACAACCAAAATCAGCTGCTAAATTC
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AATGACCCATCTACTGCTAATGCCGGGTTTGTGCCGCCAATTTCAGCAAA
ATATGCAACTCAATTTGCTACTCACAAGTCGCCACCCCAACCTATATAAA
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ATGCCGGTGCTGTACAATGTTGTCCATCCTGAAAGTGAATATTGGACCAA
TAATTCTGGTGCAGTTGCTACTACTGCTGCTGCCACTGCTCCAATGTATGA
CGCTTCTGGGTTTCCTATACCTATTAACCAATCATATATGGTAATGAATGA
ACACGAAATGGTACCTTATCAATACATGAATTCCAATGGTGCTATGATTG
GCATGATTCCACCTCACCAACAACAACAGCAACAACAACAACAATTGCC
ATGGGATATCAAAGCATGTTGAGACAACAACAACAACAACAGCAGCAGC
AGCAGCAACAACAACCTTCTTCTACAATGACTAAAAAGAAAAAGCAAAT
CCATTCGTTCAATAATAACAAGAGTTTATCATCTAATGGCGGTGGCATTAC
CAAGAAATCACACGATAATAATAATCATAGTAAAGTTAAAACTCTGTACG
GATCATTAAATGATGTTGTGAATTCCAAAGTCACCAAAGTGATCGACTCA

FIGURE 15

GATCAAGGTGGATGTGCCGGAATGATTGGTATGGTAAGCTTGCCCAATTCTTATAAGTCA
GTCTCTGTTCAAGTAAAATGGTTATATAGTGCTTAACCGGAACAAACCTTTAGCAATTGC
GATCAACTAAGATATCTTATTGTAAACCCTACCGTCATCATATGAAATATCAATCTTAGA
AATCTTAGAGAGCAATGCAAAAAAAAAAAAGAGACGAGACGGTCATCGTAAATCATGATC
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CCAGAAAAGTGCCTGTATTTAATGGTTCAAATGTTCTTTTTATAAGTACAATATCTTATC
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TATGTTTCCTGTCATTAAGAGTTTTTATTTTTTATTATAATACTCAACACGTGACTTCAG
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ACATTTGAATTCTCGTTTGGTGTTCTATTGTTAGAAGCATAAGCTTGATTAGGAAACAAC
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GACCGAGGTGTTGCTATGCTTAATGGGGATGATGCCGAAACTTTTGAGTTTACTGCTCGC
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GAAGATAATGGACTGGATACAGCCGGCTTGTTGAAACTTCCCAAATTTATACCTGGAAAA
GGGAGAGAATTCGCAGTTTTAGTGGGCGGAGCGGAATGAATATTCGGAGCAATAGTATTC
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ACGCTGTTGCCACCGAGTTTATTCGTAGACTCTACTGGAAAAGCTGAGGTGGATGCCTTT
TTTGTATTTTTCCTTACCTTTGTGGGCTGTTTAGAAGCTAAAGCGGAAGGTGATGCTCCA
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TCATTGAGCGTATTCTTGCAGTTCATTTTTTCCTTCAAATCCGAATTTTCCGCCGTAGGT
TGATTTATCCACTGCTGTTGCTGCTGTTGCTGCTGCTGCTGTTGTTGTCGCTGCTGTTCA
CTATTCGTTGCCATTGCAATATTACTCTGCAAATCTTGTTTATACTGGCTTGCCATGTAC
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CCACGGAATGCGTTTCCTCTTTATTTATGTCTATAAACGTCTTCGTGTTTATTTTTTATA
GCCTCAGTGACTTACTAACGTCAACTCACCGTGTCTTCGGCAAACAAAATGGTATCAAAG
C
AAATGCGCTTGAACAGGAGTAAAAAATGGTCACTTTTTCAATCTGTATTCTATGAATTTT

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09005

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; C12N15/81; C12Q1/68; C07K13/00
C12N1/19; //(C12N1/19,C12R1:725,C12R1:865)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	J. CELL BIOL. vol. 115, no. 3, 1991, ROCKEFELLER UNIV. PRESS, NY, US; page 407A P.O. LJUNGDAHL ET AL. 'SHR3 encodes an endoplasmic reticulum protein required for aminoacid transport in yeast' Abstracts of papers presented at the thirty-first annual meeting of the american society for cell biology, Boston, Massachusetts, USA; December 8-12, 1991; abstract no. 2362; abstract --- -/--	38-43

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 FEBRUARY 1993	23. 02. 93
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	HORNIG H.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	CELL vol. 68, no. 6, 20 March 1992, CELL PRESS, CAMBRIDGE, NA.; pages 1077 - 1090 C.J. GIMENO ET AL. 'Unipolar cell divisions in the yeast S. cerivisiae lead to filamentous growth: Regulation by starvation and RAS' see page 1079, right column, line 10 - line 13 see page 1086, left column, line 53 - right column, line 6	44,45

INTERNATIONAL SEARCH REPORT

1. ...rnational application No.
PCT/US92/09005

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: (7,8,11) incomplet.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The open reading frames for the two proteins CPH1 and PDH5 are missing in fig. 14 and fig. 15, and in respect of a possible exon-intron structure of the genes encoding the proteins PHD5 and CPH1, the cited claims were searched incompletely.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FIG. 1A

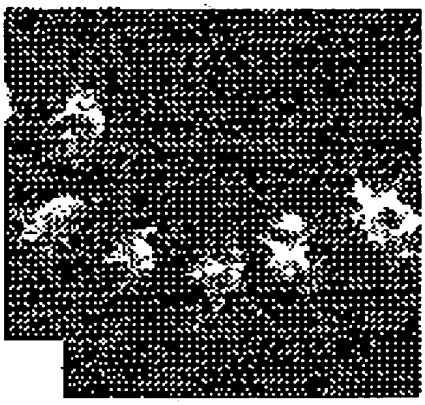


FIG. 1B

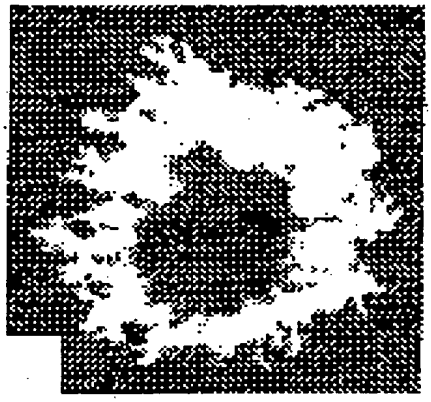


FIG. 1C

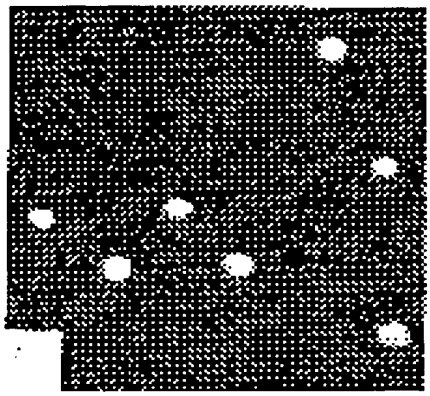


FIG. 1D

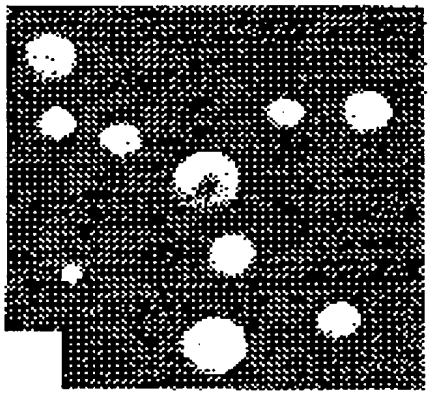


FIG. 1E

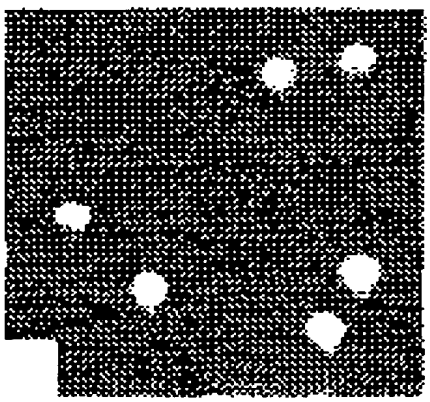


FIG. 1F

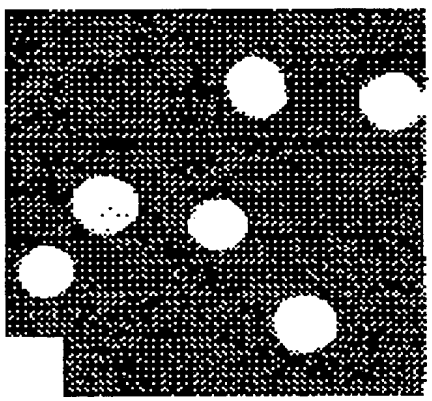


FIG. 2B

FIG. 2A

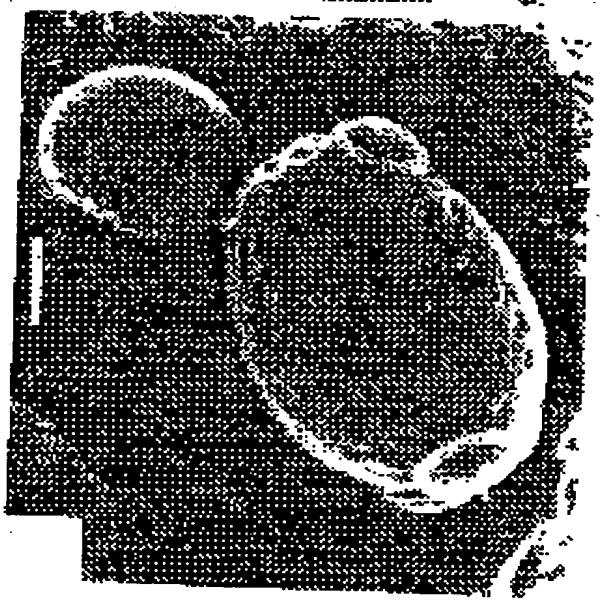


FIG. 3B

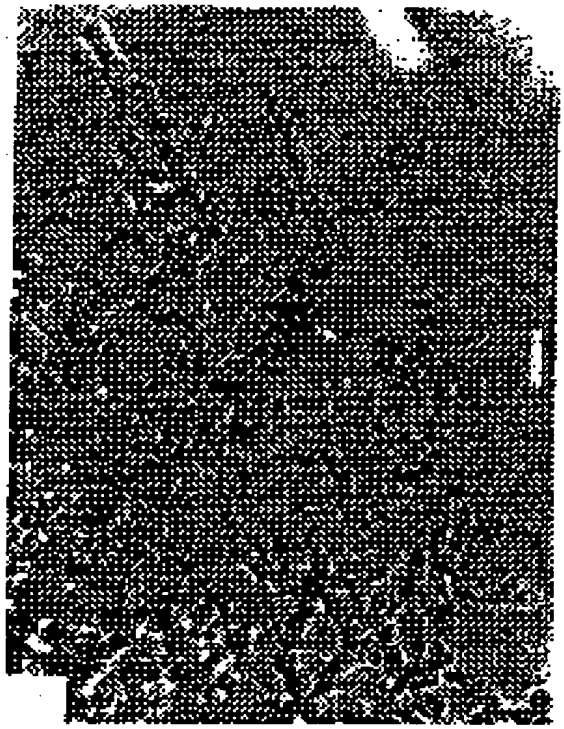
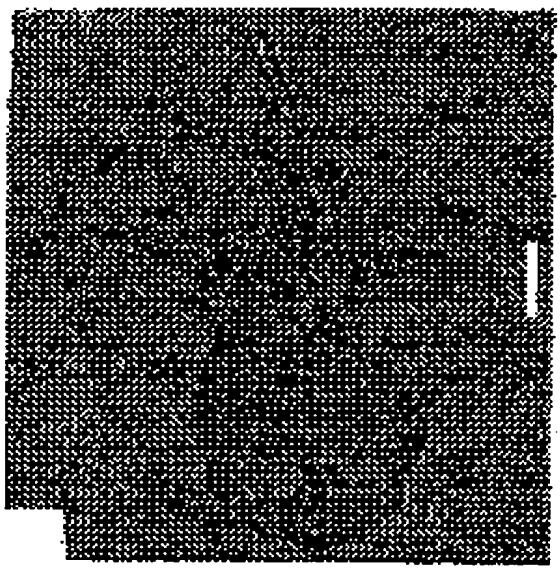


FIG. 3A



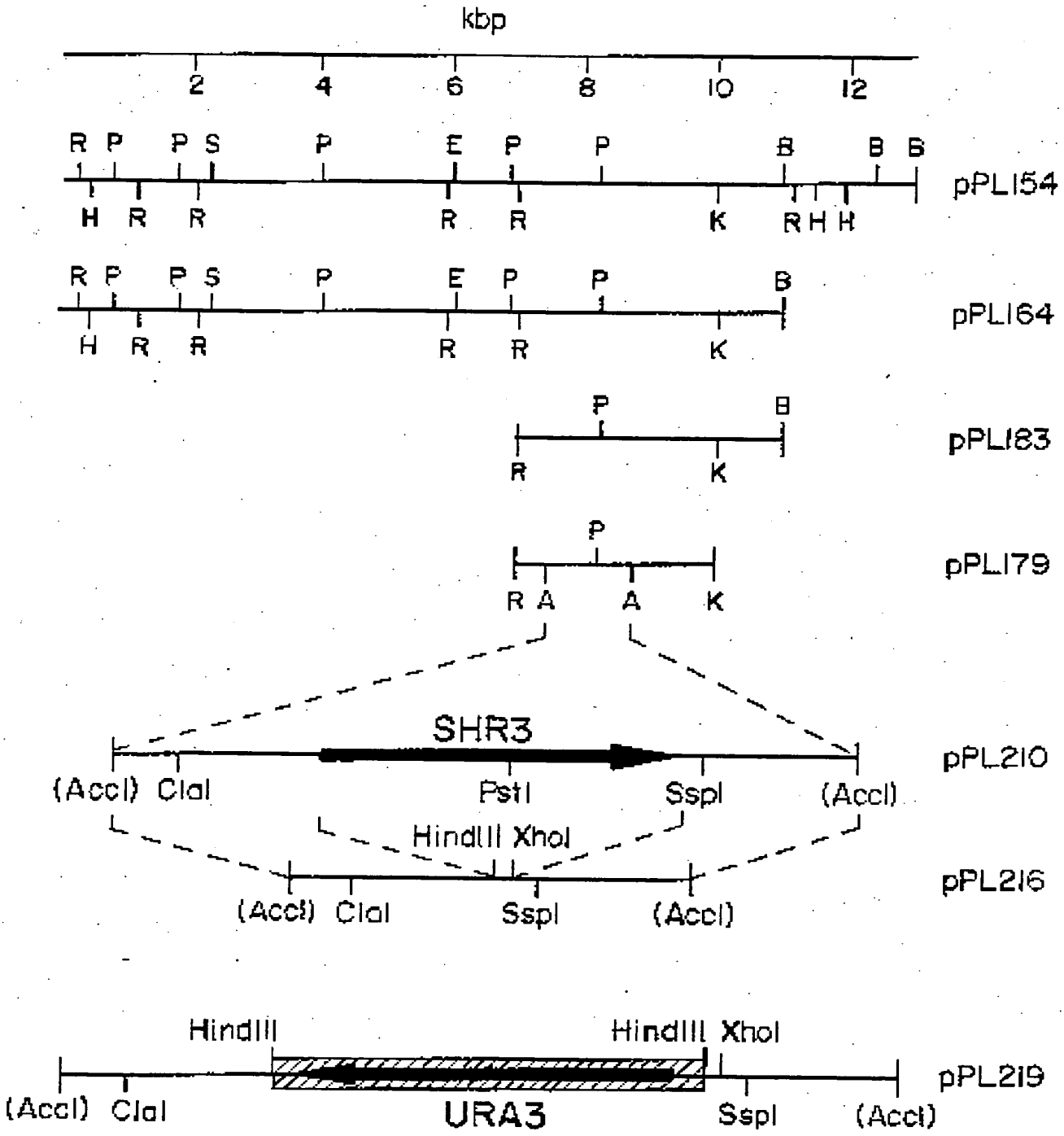


FIG. 4

SHR3

```
   1  GTAGACGTCTTCAATCTCTATCCTGTCCTCTACATTAGATTACTTTCTCATTTTCTCAGA   60
  61  AAACCTTTTAAGCTCATCGATTAAGAAGAAAAAAATCTTAGAAAATTTTCAGGAATGCCG  120
 121  GGTAAACGTATGGGGAAAGGACTTGGTGAATCGAGTTAATGTGGAGTTCGTAATAATAGT  180
 181  ACTCTCATACCAAGTTTTCCATCCATAATTGTTTTTGGTTGCCGTTACTTTACACCATCA  240
 241  CCTTTCGCCAATGTAATTGGTGGCTCTTTCCCTCTCCATCTTTTTTTATAATGTACTAAA  300
 301  ATTAATACACGATAACAATTTTTTCGGGTTGTGTAAAAACTCTGAAAATATAAAAAGTT  360
 361  GATGAAAGGACAGATTTAAGTATTTTGGAAGACAGCCGTAAATTCATTGGACTACAGAGC  420
 421  ATAAAATCCCGTGATACGATAAACTCTAACGATGTTCTCATATTCAGATTTCTGTTCTA  480
                                   M  F  S  Y  S  D  F  C  S  I
 481  TCGGTACGGCCATGATCTTATCGGCCACAACGTTCCTAATGGGTGTTTTCTTCAGTAACA  540
        G  T  A  M  I  L  S  A  T  T  F  L  M  G  V  F  F  S  N  M
 541  TGCCATACGATTATCATCTTTTATTTAATCCTAACTCTACTCAAGAGCATTTCGATTTGG  600
        F  Y  D  Y  H  L  L  F  N  P  N  S  T  Q  E  H  F  D  L  A
 601  CACTGAGACATTACCAAATTTTACATGAGACTCCATTGCCCGTGATTGTTACTTTGTGCG  660
        L  R  E  Y  Q  I  L  H  E  T  P  L  P  V  I  V  T  L  C  V
 661  TTGTTGCCGGTATTGGCTTAGTTGGTGGTATCAATTAAGGTTTTCAAGCCAAACCCTGAAC  720
        V  A  G  I  G  L  V  G  G  T  I  K  V  F  K  P  N  P  E  L
 721  TGCAGATGTTTGAGTATTGTTCATTGGGGTTGTACGTGTTGGCTATCTGTGTGTTTCTCA  780
        Q  M  F  E  Y  C  S  L  G  L  Y  V  L  A  I  C  V  F  L  T
 781  CTAATGTGAAAACAGGTATCGACTGTTCTGTGAGCCATAATTGGGAGAAGTTACGAAATC  840
        N  V  K  T  G  I  D  C  S  V  S  H  N  W  E  K  L  R  N  Q
 841  AGGGTTTGGCAGTTATTGCTTCCTCCAACATAATTTTATTAGTTATGTTTGCCGGTGTTA  900
        G  L  A  V  I  A  S  S  N  I  I  L  L  V  M  F  A  G  V  I
 901  TCATCCTACAAATTGGTTTGTGGTACAGTAACTGGGATTTGCAAAAAGATTAAAGGAGT  960
        I  L  Q  I  G  L  W  Y  S  N  W  D  L  Q  K  R  L  K  E  F
 961  TTTATGCTCAAGAAGAAAGAGAAGCTGCCAATGCCGGTAAAAAGACTGAGAAAGTTGACA 1020
        Y  A  Q  E  E  R  E  A  A  N  A  G  K  K  T  E  K  V  D  N
1021  ATGCGAAAAAGAATGATAACAAATCTAAAGGTGCTCAAAAGAGGAAGAACGCCAAAAAAT 1080
        A  K  K  N  D  N  K  S  K  G  A  Q  K  R  K  N  A  K  K
1081  AGGCCATAATATTTTTAACATTTTCTCTTTTTGTCCCTCTTTTCCCGTCTGTGACGAAAC 1140
1141  AGACGATTATACGAATCTAGTTGTATAGTAAGATCAATATTTATTATATAAATACTGTTG 1200
1201  AATAAATAAAACTCTATCTGCTTCGTGCATAAATTTACAAATAATTCCAAAGAAATAGCA 1260
1261  ACAAGTACCAGCCTTGTTTAGAAGTACGAAAATCTTGGAATCCACTTTTTTCTATTTTTA 1320
1321  AATAATTTCTCTCAAAAAGCTATTGGTTTGCTGAATATTTGGCCGAGTAATAGTAGAC  1378
```

Figure 5

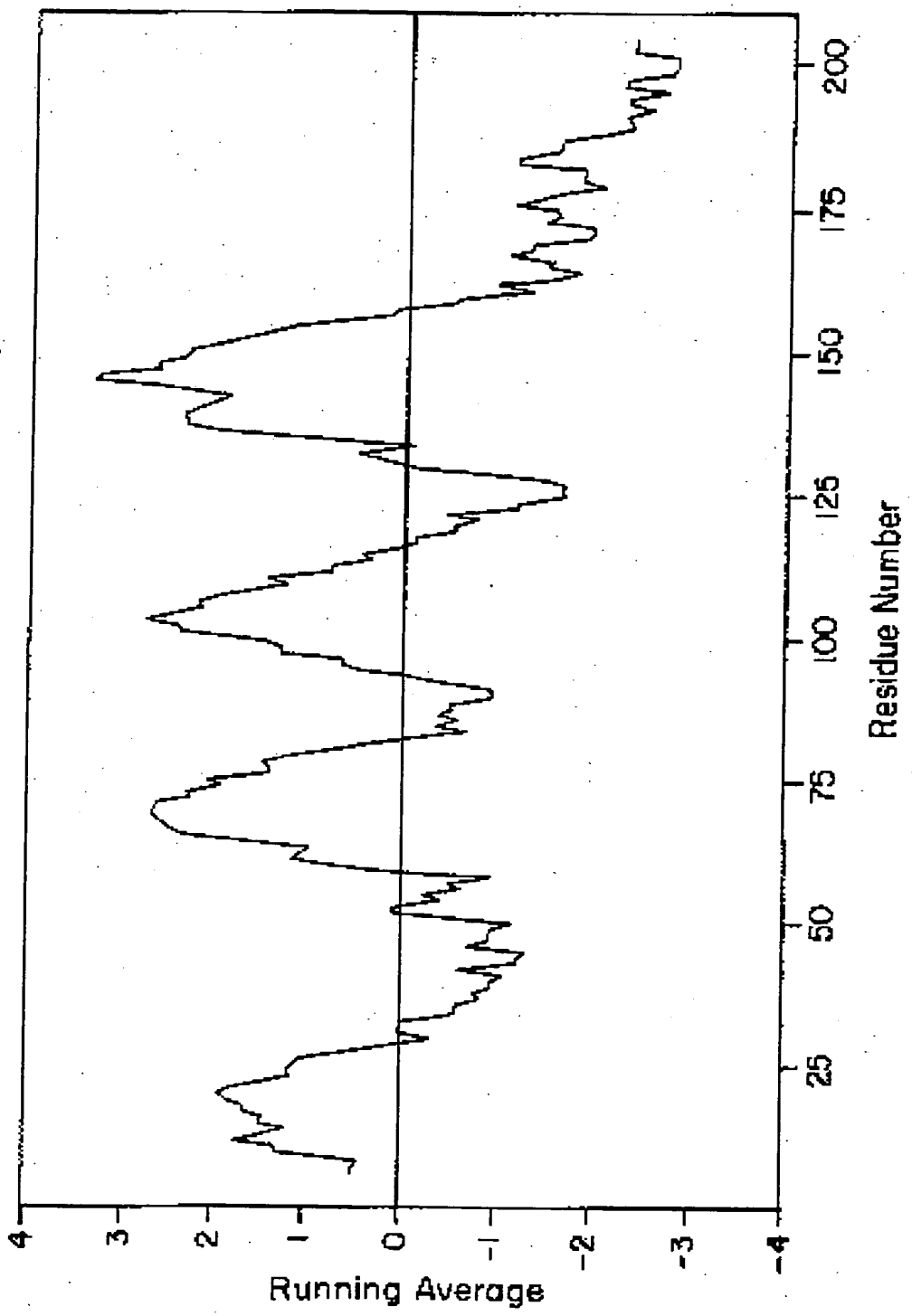


FIG. 6

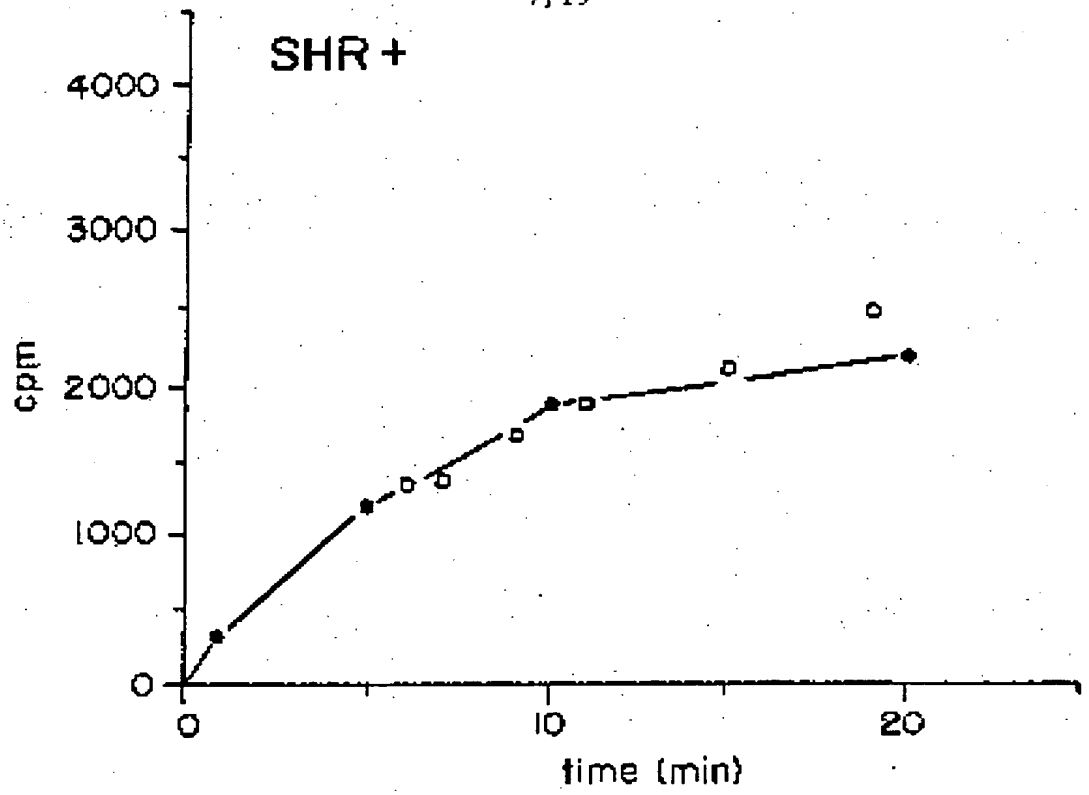


FIG. 7A

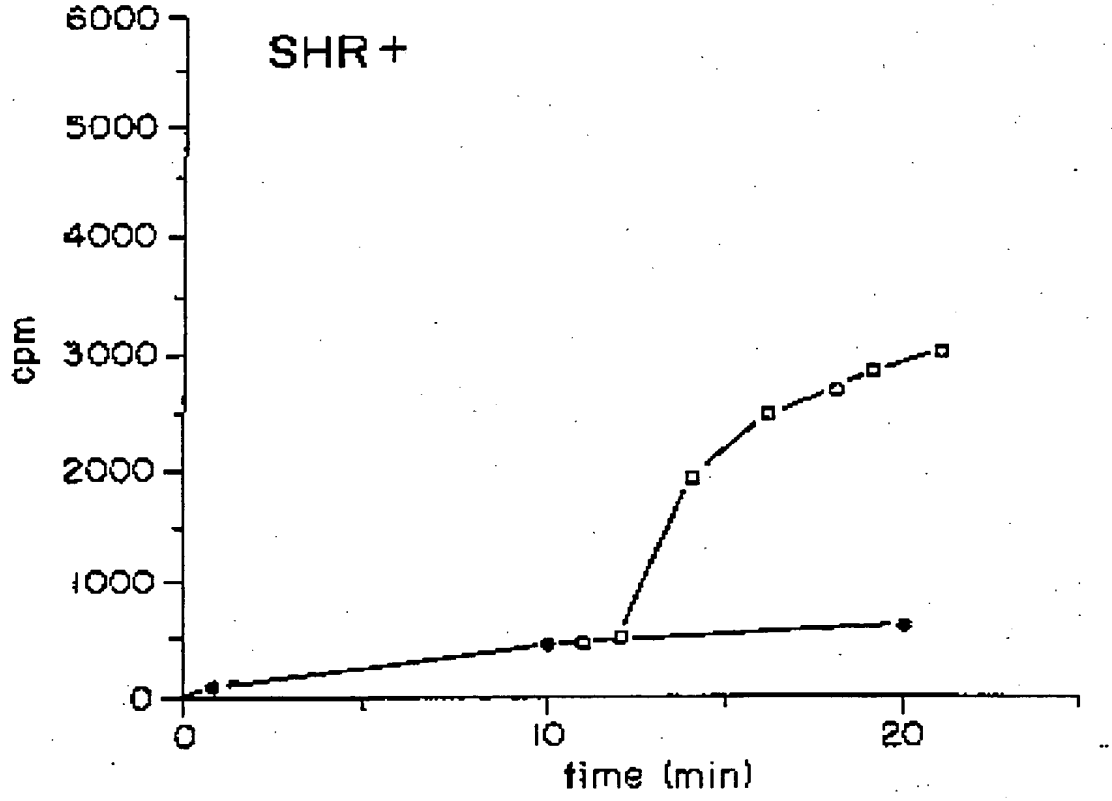


FIG. 7B

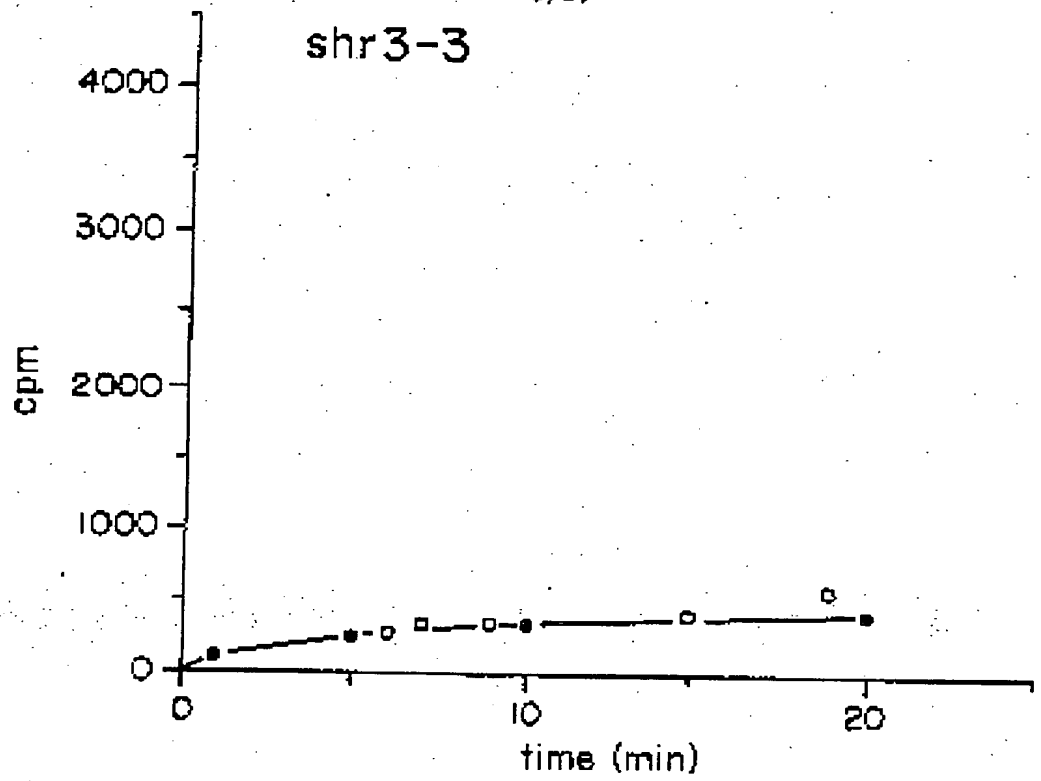


FIG. 7C

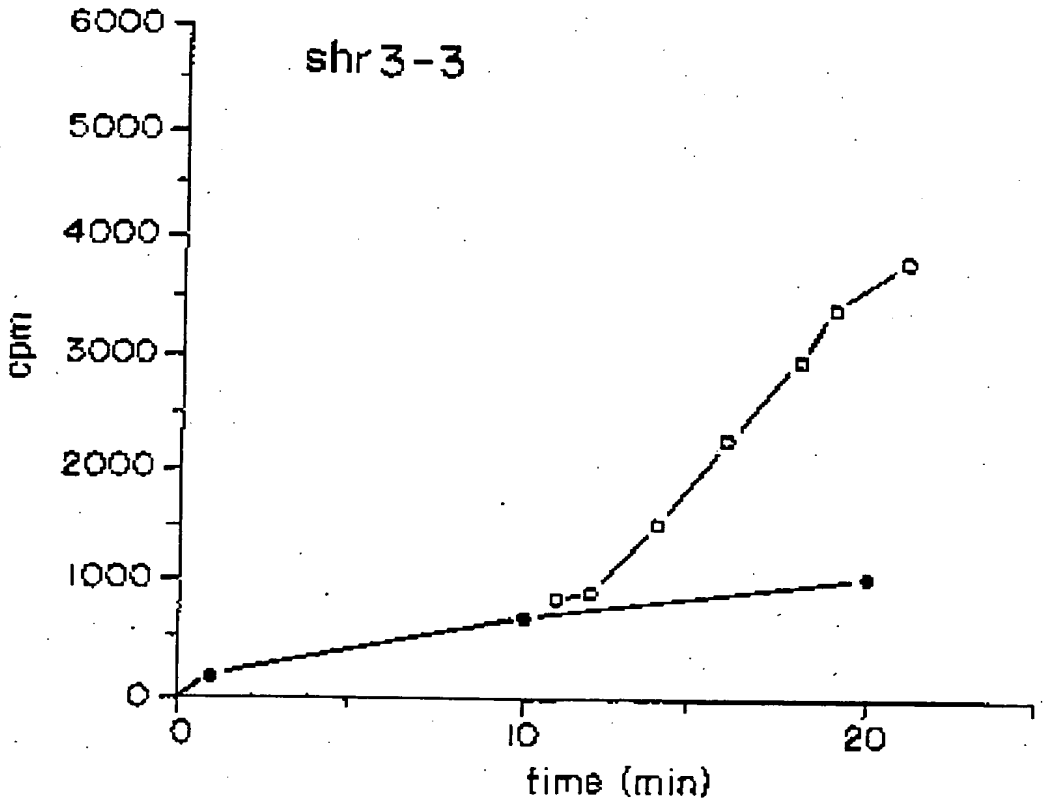


 FIG. 7D

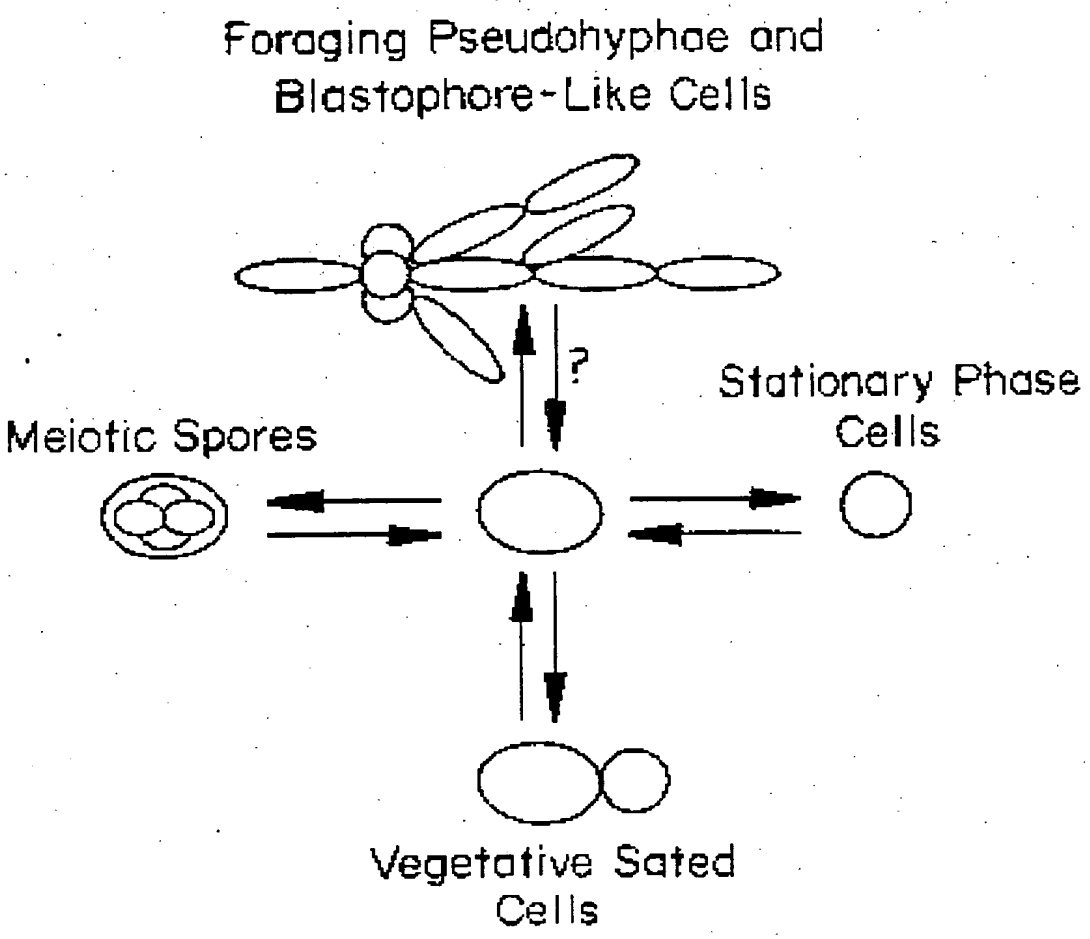


FIG. 8

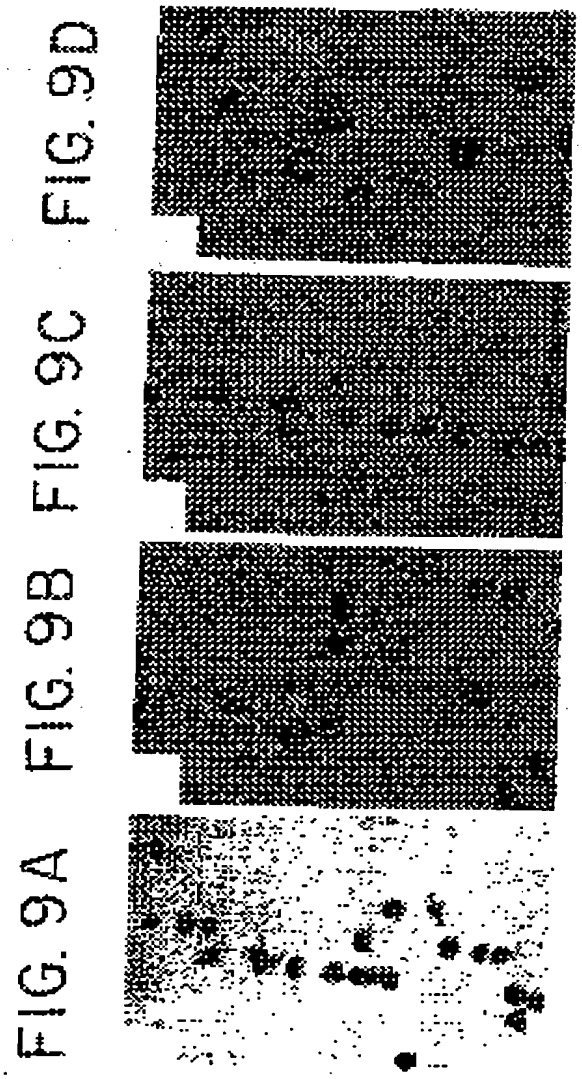


FIG. 9A FIG. 9B FIG. 9C FIG. 9D

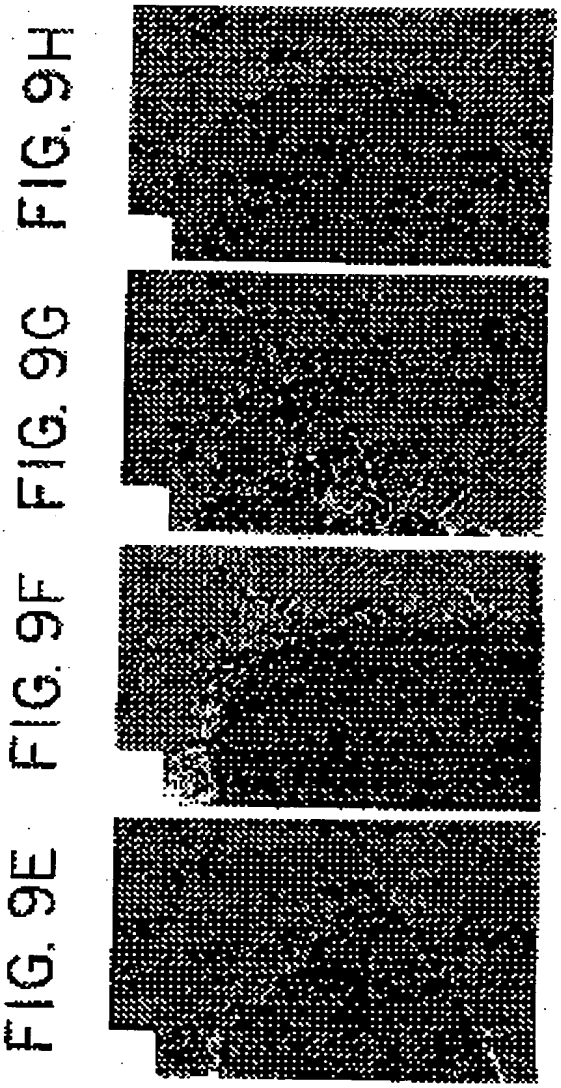


FIG. 9E FIG. 9F FIG. 9G FIG. 9H

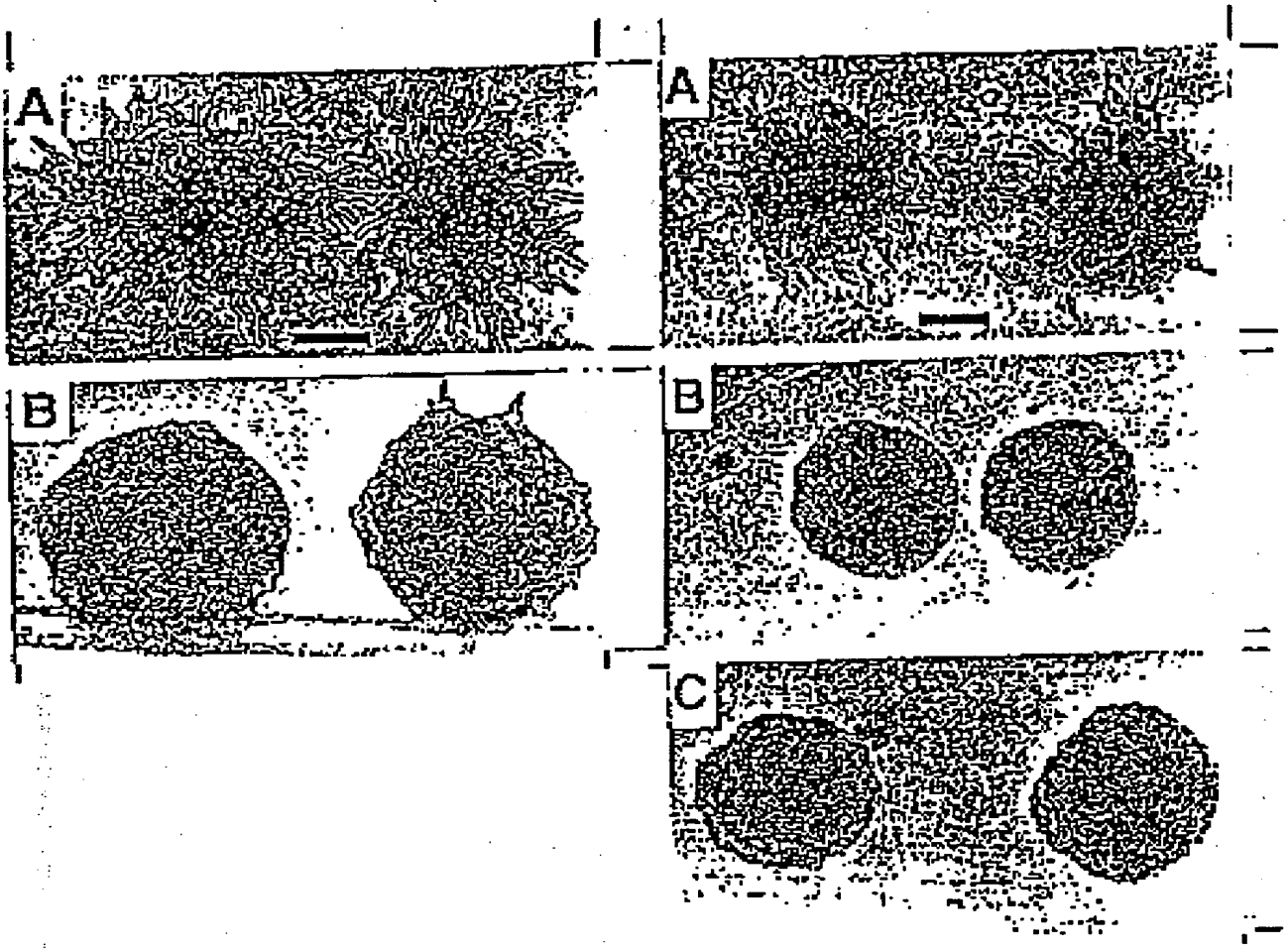


Figure 10

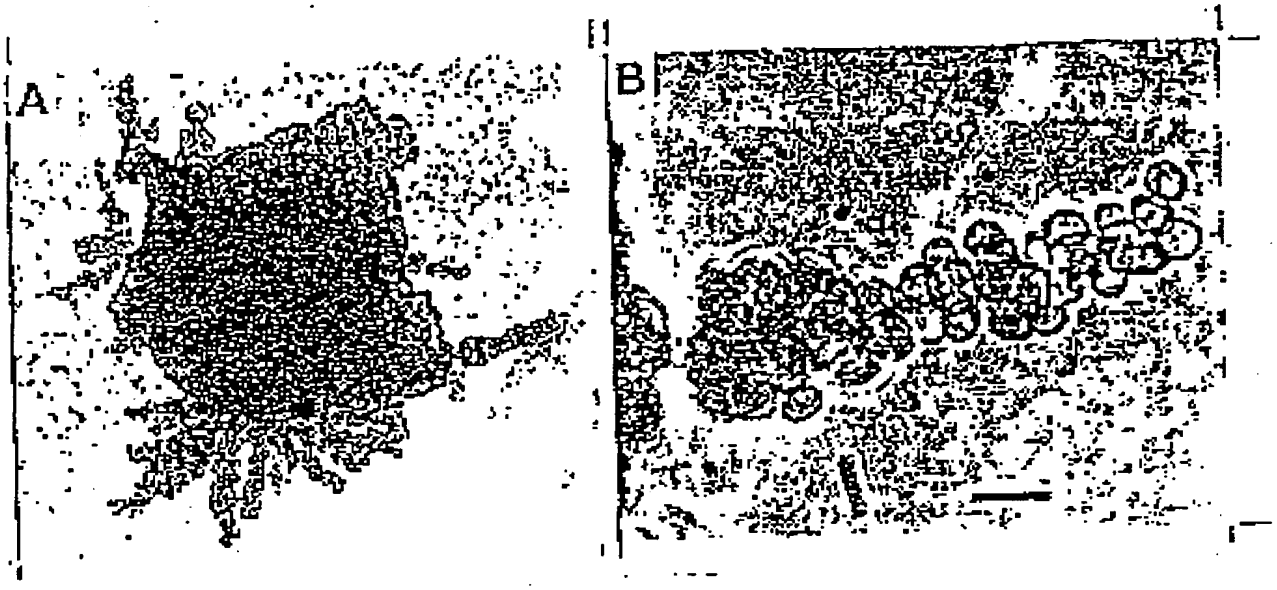


Figure 11

FIG. 11B

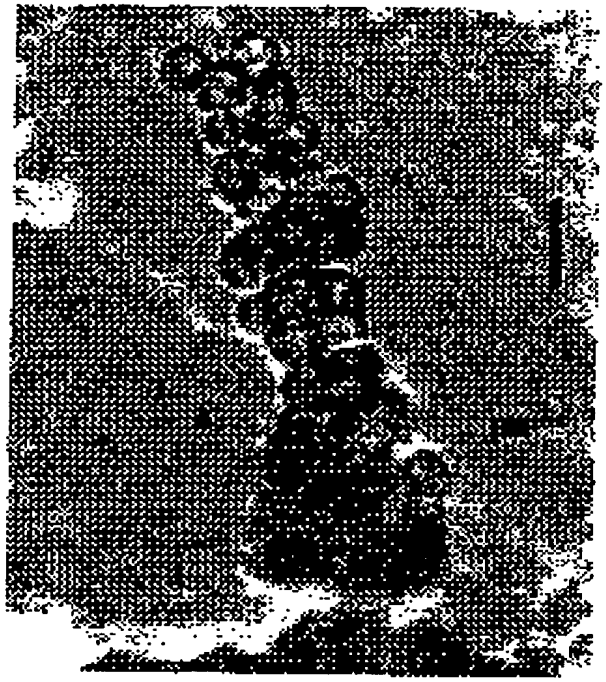


FIG. 11A

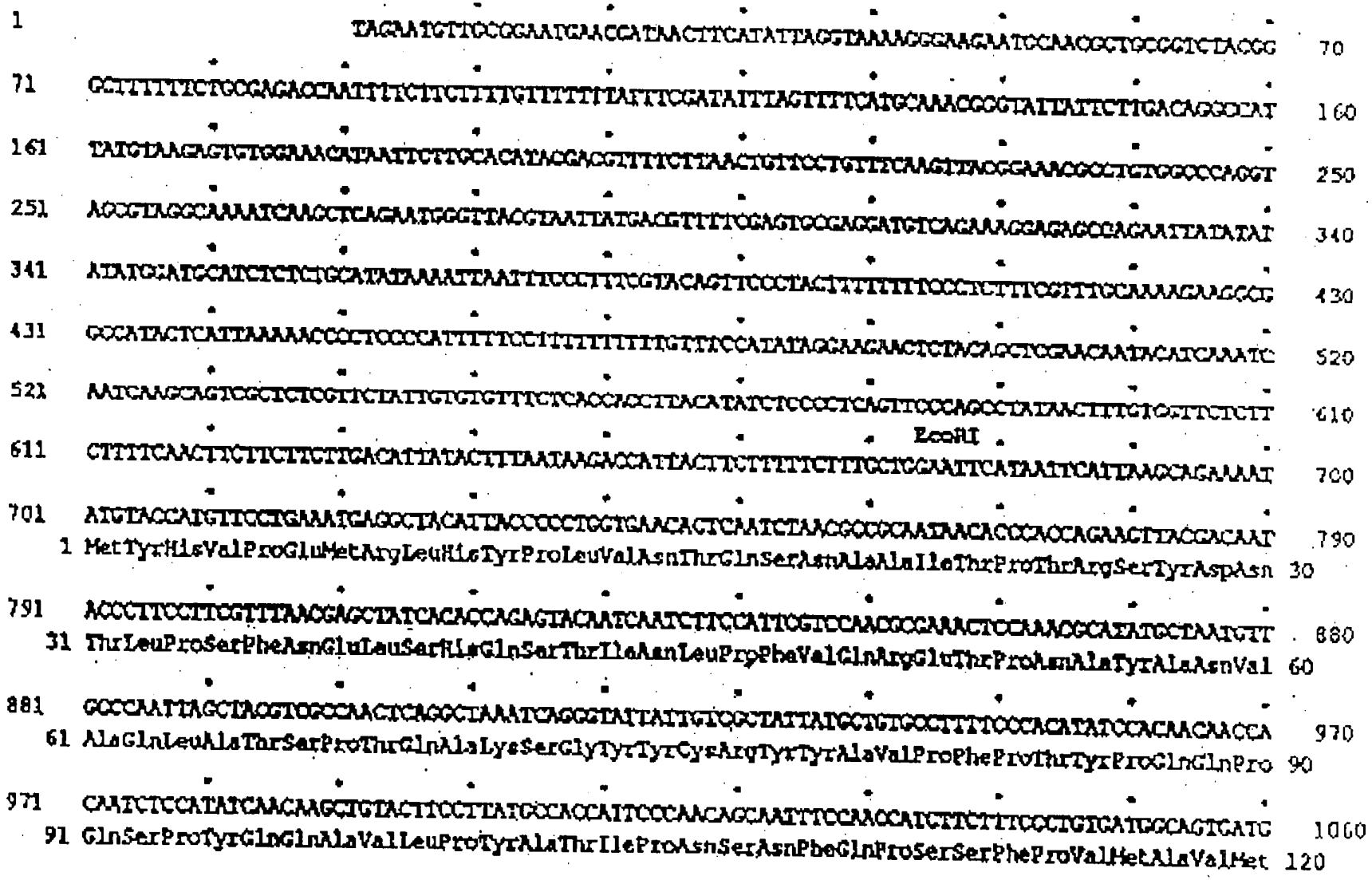


FIGURE 12A

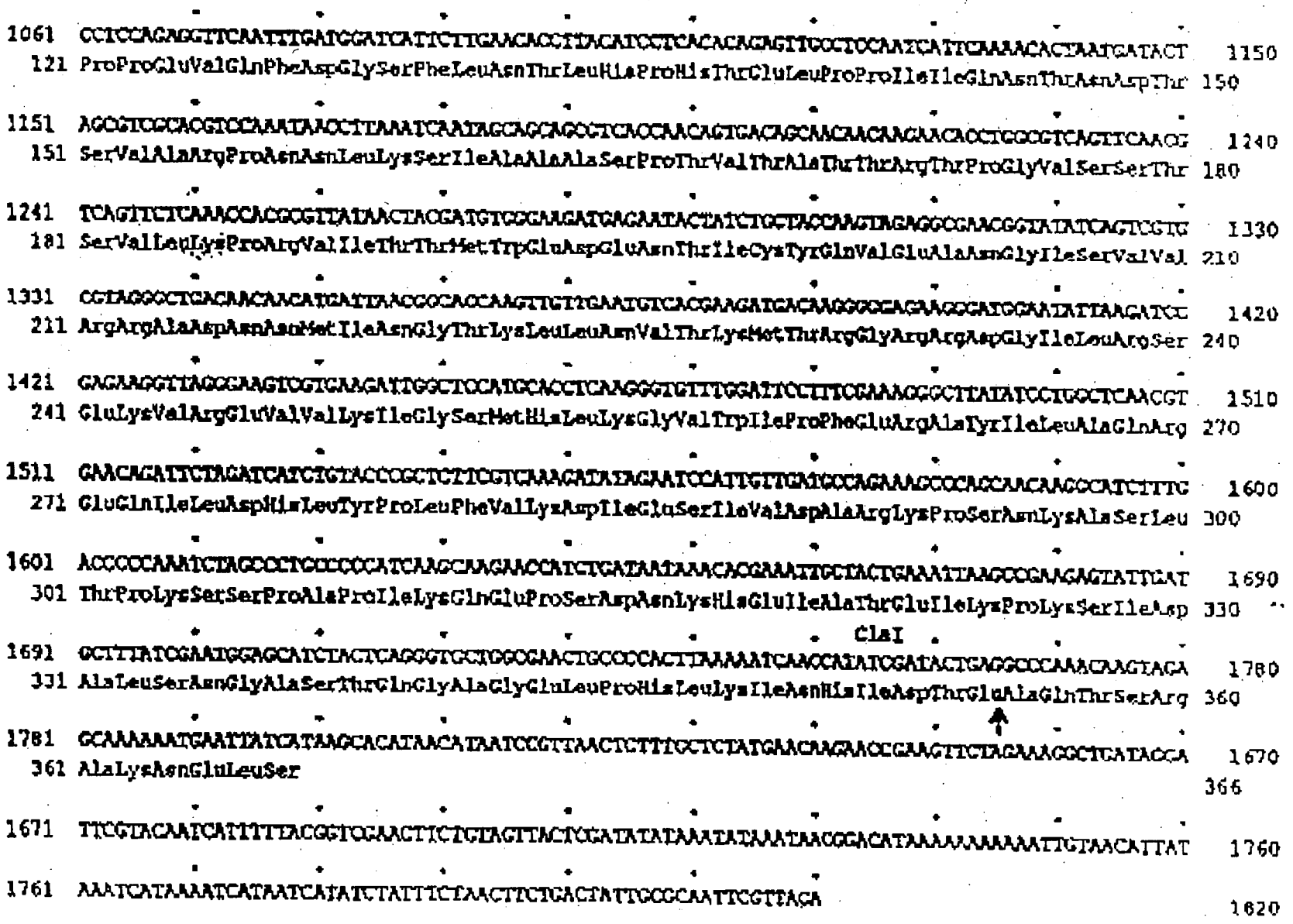


FIGURE 12B

```
PHD1  184  KPRVITTMWEDENTICYQVEANGISVVRRADNNMINGTKLLNVTKMTRGRRDGILRSE
stuA  127  ----TA-L-----GSL--------K-VC-A---E---G---------------AG-------------K--
SWI4   39       -A-YS-TDVYE---IRGFXTKI-N---IKDDW---I-QVFKIAQFSKIK-TK---EK-SND
cdc10 134

      KVREVVKIGSMHLKGVWIPFERAYILAQREQILDHLYPLFVKDIESIV  289 PHD1
      ---N-------P-------------D---LEF-NK-K-T-L---------QH-SNLL  232 stuA
      MQH-K-QG-YGRFQ-T----LDS-KF-VNKYK-I-                        129 SWI4
                QID-----V-YD---ISI-K-YGVYKI-Q--                  161 cdc10
```

FIGURE 12C

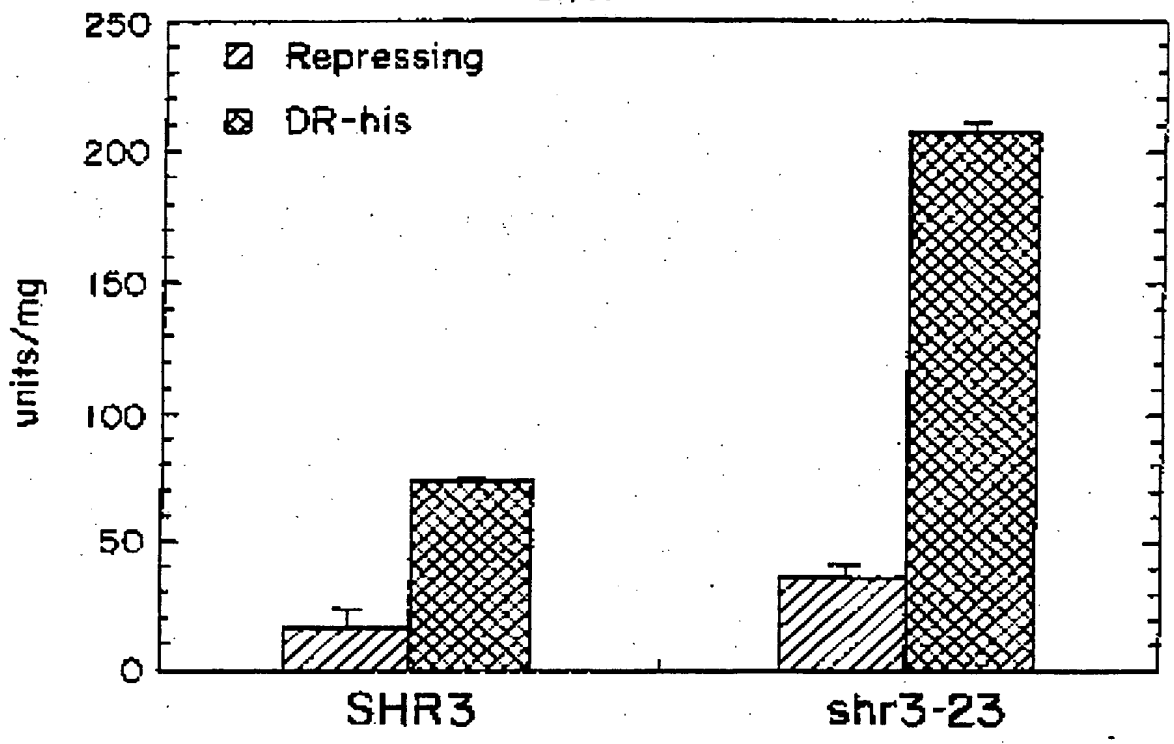


FIG. 13A

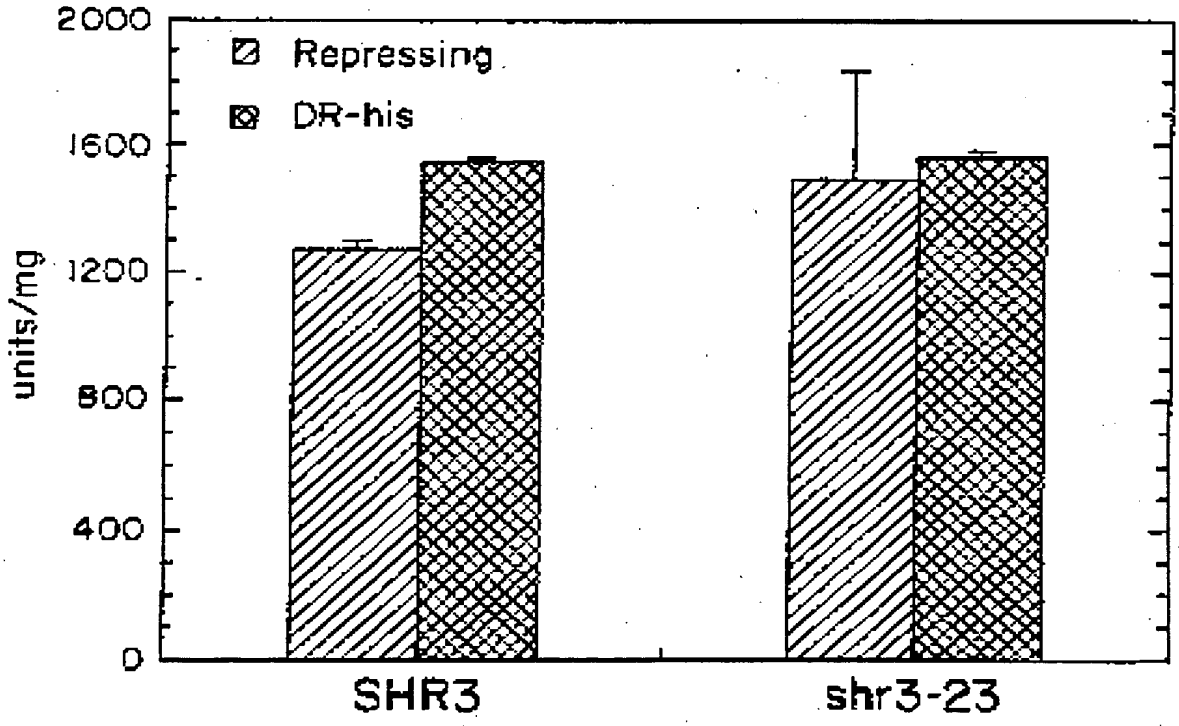


FIG. 13B

FIGURE 14

CATTAATTCTTAGTTAAATATATTTCTTTTCACTTTTTTCACTTTTTTCATTTT
TCAATTGTTGTGCCCCCCCCTCGTTGCGGATCAAATAATATAACTGCCTAA
TACACTCTTTCGCCATGTCAATTACTAAAACATACAATGGTGATCCTACAT
CACTTGTACCAACTCAACTGGTAAAAGAATCATTAAGACTAATTGAAGAT
TTAAAATTCTTTTTAGCCACTGCTCCAGCAAATTGGCAAGAGAATCAAGT
GATTCGAAGATACTATTTAAACCACGATGAAGGTTTTGTCAGTTGTGTTTA
TTGGAATAACTTATATTTTATCACTGGTACCGATATCGTCCGTTGTATCGTT
TATAAATTTGAACATTTTGGAAGGAAAATCATTGATCGGAAAAAGTTTGA
AGAAGGTATTTTCTCCGATTTAAGAAATTTGAAATGTGGCGCCGATGCAA
TTTTGGAACCTCCTCGTTCAGAGTTTTTAGAATTCTTATTTAAAAATTCTTG
TTTGAGAACACAAAAGAAACAAAAAGTGTTTTTTTGGTTTAATGTTCCTCA
TGATAAGTTAATGGCAGATGCCTTGGAACGAGATTTGAAAAAGGAAAAA
ATGGGTCAAAGACCAACAACAATGGCCCATCGAGAACCAGCATTATCAT
TCCATTACGACGAGTCTTCCAGTTTATACACTCAATTGGGTAAACACATGG
AAACTCAGAAGAGAATCAACGACGCAGCCACAAGCTCTACTTCTAATACT
GCTACCACTTTAACCGACACCGGTGTATCCTCAGGATTGAATAATACCAC
TTCTGGTGGCGGCAGTGATAGTGCAACCTCCACACACAACAACAATGAG
GCATCGACCAAACCAAGTAATGGCAGTGAAAAATCGTCACCGGAGTACA
CTACAACTGCCCGCGGTAGAGATGAGTTTGGATTCCTTAATGAAGCCACA
CCAAGTCAATACAAAGCCAATTCAGATTATGAAGACGATTTCCCATTGGA
TTATATCAATCAGACCACTCAAAATTCTGAAGATTATATTACTTTGGATGC
AAATTATCAGGCAGGAAGTTATGCAAATATGATCGAAGACAATTACGATT
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GTACAGCTGCGACTGCAACAACATCAAACCAAGTTGCCTTCAACGACGA
ATACTTGATTGAACAAGCCCAACCAATAAGGACTCCACTACCCCCAATAT
CATCATCAACAATATCCGGATTATTACAACCAAAATCAGCTGCTAAATTC
TTTTCACTACAGAGTGCTAATGGTGGAGAAGAATTTTTCCCTGCTTATCAA
AATGACCCATCTACTGCTAATGCCGGGTTTGTGCCGCCAATTTCAGCAAA
ATATGCAACTCAATTTGCTACTCACAAGTCGCCACCCCAACCTATATAAA
GGCAATACCGCAAACTGGTGCTGCTGCTGCCACTGGTAATGGTGGTCAAC
CACAACAATATTATGACCAGGCAACTGGTAATGCTTTTTACCCAGCAGAA
ATGCCGGTGCTGTACAATGTTGTCCATCCTGAAAGTGAATATTGGACCAA
TAATTCTGGTGCAGTTGCTACTACTGCTGCTGCCACTGCTCCAATGTATGA
CGCTTCTGGGTTTCCTATACCTATTAACCAATCATATATGGTAATGAATGA
ACACGAAATGGTACCTTATCAATACATGAATTCCAATGGTGCTATGATTG
GCATGATTCCACCTCACCAACAACAACAGCAACAACAACAACAATTGCC
ATGGGATATCAAAGCATGTTGAGACAACAACAACAACAACAGCAGCAGC
AGCAGCAACAACAACCTTCTTCTACAATGACTAAAAAGAAAAAGCAAAT
CCATTCGTTCAATAATAACAAGAGTTTATCATCTAATGGCGGTGGCATTAC
CAAGAAATCACACGATAATAATAATCATAGTAAAGTTAAAACTCTGTACG
GATCATTAAATGATGTTGTGAATTCCAAAGTCACCAAAGTGATCGACTCA

FIGURE 15

GATCAAGGTGGATGTGCCGGAATGATTGGTATGGTAAGCTTGCCCAATTCTTATAAGTCA
GTCTCTGTTCAAGTAAAATGGTTATATAGTGCTTAACCGGAACAAACCTTTAGCAATTGC
GATCAACTAAGATATCTTATTGTAAACCCTACCGTCATCATATGAAATATCAATCTTAGA
AATCTTAGAGAGCAATGCAAAAAAAAAAAAGAGACGAGACGGTCATCGTAAATCATGATC
ATAGTAATGTCAAAAGTTAATTTACCATTATGCCAAGCTACTTCAATGAGTGTACATCAA
CCAGAAAAGTGCCTGTATTTAATGGTTCAAATGTTCTTTTTATAAGTACAATATCTTATC
TAAAAATTAAACACTTCTATCTTATCATGGAAGAAAAACAAAGCTATCACAAAACAAATA
TCCTAAAAAACCGTTTATAAAAAGTCGTTGCTGTGAAAATGGTTCAGTTCACAGGGCTTA
AACCCATCCCCAAATTTAGCGAAATGTCAGATACGTAACTCCATTCTCCTAGCTTTTTAT
TATGTTTCCTGTCATTAAGAGTTTTTATTTTTTATTATAATACTCAACACGTGACTTCAG
CCTTCCCAATTAATAAAATTGAAATCATTTTCATTTGTATCCAGTAAATTGAGATCGTAA
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TTCTGATTTTCATTAGTACTGGTTTGCTGAGGACCCAAAGTTGTACCCACATTGGATGAG
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ACTTGCGCCTCCAGTATGTTTCCCCCATTCGGCAATTCTTGGGATACTGTATTATTCTGT
GAAGTGATTATTTTAGAGTTCTTTAAAGTAATATTCAAATTGGTGGTTGAAAACCCATGT
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GACCGAGGTGTTGCTATGCTTAATGGGGATGATGCCGAAACTTTTGAGTTTACTGCTCGC
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ATAGCGGGTTCACTTGTTGTTGAGCCTGGTGTGGTAATATTTGGCGAACAAGCGTTCGTC
GATCCCGCGGAAACTGTAGCGGTATTATTTTTTCTTCGTCTCTTAGCCACTAGACCGGCT
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GATTTGTTATTAGTTGTATTATTTGTGGTATTATTGTTACACGGGTTTCCCACTGGATTT
TCCGTAGTGGGTTGCATTGGATAATTATAGACTGGCCAGCCAGTAACGTTTTGACTCCGA
TTCTGGGTTGGCCCTACATTTGTAAAATCGCCGGAAGCGGCGCTGTGGGAGCAACAGCA
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GGGTTACCAACCATAGGAATTGGTATAGGGTTCATATTAACATTGCGCATTTGAACCGCA
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TCTGAGCCACCTCTGGAAGAACTGGTATTAAAGATGTCCCAGAATATTTGCCACCATTCA
TACAAAAAGCCTTGAGGTGTATCTACTACCTTCGAGAACGTATTCTGGTTACCATTGTTT
TCTTTAGATTTGGGTCCGTCGACTGGGTTTTGGCCTTTGTCTCTATCTAGGTGCGCATCT
TGAGCAAAGGCTGCTGCAGTGTTTTTCAAAGACGACTTCGTAAGAAAGTCAAATATGTAC
TCATTGAGCGTATTCTTGCAGTTCATTTTTTCCTTCAAATCCGAATTTTCCGCCGTAGGT
TGATTTATCCACTGCTGTTGCTGCTGTTGCTGCTGCTGCTGTTGTTGTCGCTGCTGTTCA
CTATTCGTTGCCATTGCAATATTACTCTGCAAATCTTGTTTATACTGGCTTGCCATGTAC
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CCACGGAATGCGTTTCCTCTTTATTTATGTCTATAAACGTCTTCGTGTTTATTTTTTATA
GCCTCAGTGACTTACTAACGTCAACTCACCGTGTCTTCGGCAAACAAAATGGTATCAAAG
C
AAATGCGCTTGAACAGGAGTAAAAAATGGTCACTTTTTCAATCTGTATTCTATGAATTTT